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REMARKS/ARGUMENTS

Support for probes and primers derived from gene 513 sequences as recited in amended claims 5 and 6 is provided at e.g., p.8, lines 17-24. Support for the definition of normal level of cedar pollen specific IgE in claims 5 and 6 is found in page 26, line 4 of the specification. Support for new claims 10 and 11 is provided at e.g., p. 9, line 17. No amendment should be construed as acquiescence in any ground of rejection. Applicants respond to the outstanding office action using the paragraph numbering of the office action.

2. Claim Rejections under 35 USC § 112, first paragraph

Claims 4 to 8 are rejected under 35 USC §112, first paragraph, for being beyond the scope disclosed in the specification. The Examiner states that the present claims encompass the use and detection of sequences that are not described in the specification, such as full-length cDNAs and genomic DNAs. The Examiner asserts that, despite the broad scope of the claims, the specification only discloses the partial cDNA of SEQ ID NO:1.

In response, claim 5 has been amended to recite a "probe comprising nucleotides of gene 513, wherein the nucleotides consist of a segment of nucleotides between positions 36 and 1171 of SEQ ID NO:1 or the complement thereof" and claim 6 has been similarly amended to recite a primer that comprising nucleotides of gene 513, wherein the nucleotides consisting of a segment of nucleotides between positions 36 and 1171 of SEQ ID NO: 1 or the complement thereof. New dependent claims also limited by the term "consists of" have been added. Claim 4 has been canceled.

The first transitional term "comprising" in claims 5 and 6 leaves the probe or primer open to sequences unrelated to gene 513. It is appropriate that the probes remain open to inclusion of unspecified flanking sequences unrelated to gene 513 in that the use of such sequences for purposes such as probe immobilization or detection is conventional in the art. Applicants are in possession of flanking sequences suitable for e.g., immobilization or detection, as is any other person of skill in the art.

The embedded transitional term "consisting of" in claims 5 and 6 requires that the nucleotides of gene 513 present in the probe or primer be part of a segment within positions 36 and 1171 of SEQ ID NO:1, or the complement thereof. The Examiner's concerns with respect to the claimed probes or primers including regions of gene 513 beyond those specifically contained in SEQ ID NO:1 are moot because the claims no longer include such regions.

3. Claim Rejections under 35 USC § 112, second paragraph

Claims 4 to 9 are rejected under 35 USC §112, second paragraph. The Examiner alleges that claim 4 does not recite a "positive process step" that results in the detection of SEQ ID NO: 1. The Examiner also states that claims 5 to 9 contain a relative definition of "normal", and that this term is therefore unclear. In response, claims 5 and 6 have been amended to define "normal level" by replacing this term with the term "3.5AU/mL or less." Claim 4 has been canceled.

5. Claim Rejections under 35 USC § 102

Claim 1 is rejected under 35 USC § 102(b) as being anticipated by Koch (US6,610,481). The Examiner states that Koch et al. disclose a nucleic acid sequence that is identical to that of SEQ ID NO: 1. Claim 1 has been canceled.

6. Claim Rejections under 35 USC § 112, first paragraph

Claims 1 and 4 to 8 are rejected under 35 USC 112, first paragraph, as containing subject matter that is not described in the specification. In the previous response, dated January 28, 2004, these claims were amended to remove nucleotides 1-35, a Marathon kit adaptor sequence, from SEQ ID NO: 1. However, the Examiner rejects this amendment, alleging that the specification does not show basis for the positions 1-35. The Examiner also asserts that the user manual provides two primers AP1 and AP2, and that it is not clear to which the nucleotides 1-35 correspond. This rejection is respectfully traversed.

The first 35 nucleotides of SEQ ID NO:1 are identical to the 23 nucleotides of Nested Adaptor Primer 2 of the Marathon kit plus 12 nucleotides from the Marathon cDNA Adaptor immediately 3' prime to the binding site for Nested Adaptor primer 2 (see below)

AP2 primer

Adaptor

ACTCACTATA GGGCTCGAGC GGC CGCCCCGGG CAGGT

ACTCACTATA GGGCTCGAGC GGC CGCCCCGGG CAGGT **SEQ ID NO:1**

The present specification discloses the cloning of SEQ ID NO:1 using a Marathon cDNA Amplification Kit. According to the user manual (attached), the Marathon cDNA Adaptor is ligated to the 5' end of a target sequence (see page 53 of user manual). The AP2 primer is used as an inner primer to perform "nested" PCR (see page 32 of user manual). The combination of attaching the Adaptor to a cDNA of interest and then amplifying the Adaptor using nested Adaptor 2 generates a cDNA in which the first 23 nucleotides are those of the AP2 primer and the next 12 are those of the Adaptor. Therefore, given the teaching of the specification that SEQ ID NO:1 was prepared using the Marathon cDNA kit, and given the fact that the first 35 nucleotides of SEQ ID NO:1 are identical to the first 35 nucleotides that would be generated from using the Marathon Adaptor and AP2 primer, a skilled person would understand that applicants were in possession of a cDNA sequence beginning at nucleotide 36 of SEQ ID NO:1.

Appl. No. 10/019,832
Amdt. dated July 19, 2004
Amendment under 37 CFR 1.116 Expedited Procedure
Examining Group

PATENT

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 650-326-2400.

Respectfully submitted,



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CLONTECH

Innovative
Tools to
Accelerate
Discovery

Marathon™ cDNA Amplification Kit User Manual

PT1115-1 (PR15735)

Published 08 June 2001

Catalog #: K1802-1

See List of Components for storage conditions

FOR RESEARCH USE ONLY

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Suppression PCR is covered by U.S. Patent #5,565,340. Foreign patents pending.

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I. Introduction & Protocol Overview

Marathon™ cDNA amplification is a method for performing both 5' and 3' rapid amplification of cDNA ends (RACE) from the same template (Chenchik *et al.*, 1995; 1996; Figure 1). This technology has been cited on over 140 research articles. For a complete list of citations, see CLONTECH's web site (www.clontech.com). The method is made possible by CLONTECH's patented suppression PCR technology (Siebert *et al.*, 1995) and other innovations in the design of the Marathon Adaptor (see Appendix A). When compared to conventional kits used for 5'-RACE, Marathon RACE reactions are more efficient and reproducible with considerably less smearing and fewer false bands. The Marathon protocol utilizes CLONTECH's Advantage 2® Polymerase Mix, which is ideally suited for long-distance PCR (LD PCR; Barnes, 1994; Cheng *et al.*, 1994), and has been extensively tested in the Marathon procedures. Therefore, Marathon RACE reactions are capable of amplifying much larger templates than can be amplified with conventional RACE methods. Furthermore, given the lower rate of misincorporation observed with LD PCR, Marathon RACE products should have higher fidelity to the sequence of the original RNA. Additionally, the Marathon adaptor-ligated double-stranded (ds) cDNA can be used to amplify cDNAs for many different genes.

Marathon cDNA amplification is a flexible tool—many researchers use this kit in place of conventional RACE kits to amplify just the 5' or 3' end of a particular cDNA. Others perform *both* 5'- and 3'-RACE, and many then go on to clone full-length cDNAs using one of the two methods described in the latter part of the protocol. In many cases, researchers obtain full-length cDNAs without ever constructing or screening a cDNA library.

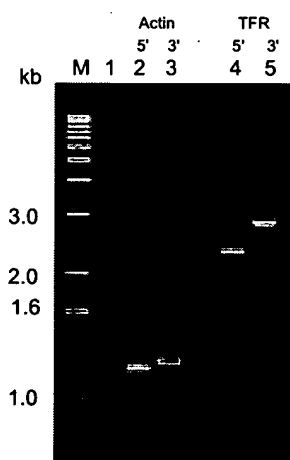


Figure 1. Typical Marathon 5' - & 3'-RACE results. 1 µg of poly A⁺ human placental RNA was used as the starting material. Lane 1: Control reactions primed with AP1 alone. Lane 2: 1.2-kb 5'-RACE product generated with actin primers. Lane 3: 1.3-kb 3'-RACE product generated with actin primers. Lane 4: 2.6-kb 5'-RACE product generated with TFR primers. Lane 5: 2.9-kb 3'-RACE product generated with TFR primers. Lane M: DNA size markers.

I. Introduction & Protocol Overview *continued*

The only requirement for designing a Marathon cDNA amplification experiment is that you know at least 23–28 nucleotides (nt) of sequence information in order to design gene-specific primers (GSPs) for the 5'- and 3'-RACE reactions. (Additional sequence information will facilitate analysis of your RACE products.) This minimal requirement for sequence information means that Marathon cDNA amplification is well suited for characterizing RNAs identified as expressed sequence tags (ESTs; Sikela & Auffray, 1993) or by methods such as differential display (Liang & Pardee, 1992) or RNA fingerprinting (Welsh *et al.*, 1992; Welsh *et al.*, 1994). In particular, Marathon cDNA amplification is an excellent tool for cloning full-length cDNAs corresponding to differentially expressed mRNAs identified with the CLONTECH PCR Select™ cDNA Subtraction Kit (#K1804-1) or the Delta™ Differential Display Kit (#K1810-1). The Marathon Kit can also be used to obtain full-length clones of partial cDNAs obtained through library screening.

TABLE 1: EXAMPLES OF cDNAs AMPLIFIED BY MARATHON RACE

Gene ^a	RNA	Abundance of mRNA	Size of amplified cDNA (kb)
Transferrin receptor	Placental poly A ⁺	Low-med.	5.1
Actin	Placental poly A ⁺	High	1.9
Rat lung-specific protein	Lung poly A ⁺	High	2.1
Inducible nitric oxide synthase	Placental poly A ⁺	Low-med.	4.1
GCSF receptor	Thymus poly A ⁺	Low-med.	2.9
Insulin-like growth factor receptor type 1	Thymus poly A ⁺	Low-med.	5.1
Insulin-like growth factor receptor type 2	Thymus poly A ⁺	Low-med.	8.9
HIV-induced genes 1 & 2 identified by differential display	HIV-infected macrophage poly A ⁺	Med.	0.7, 1.7 ^b
Interferon- α	Placental poly A ⁺	Low	2.7
G3PDH	Placental poly A ⁺	High	1.5
β_2 -microglobulin	Placental poly A ⁺	High	0.6

^a Human unless otherwise indicated.

^b When conventional RACE failed to produce the 5' ends of these two genes, Marathon RACE was used.

I. Introduction & Protocol Overview *continued*

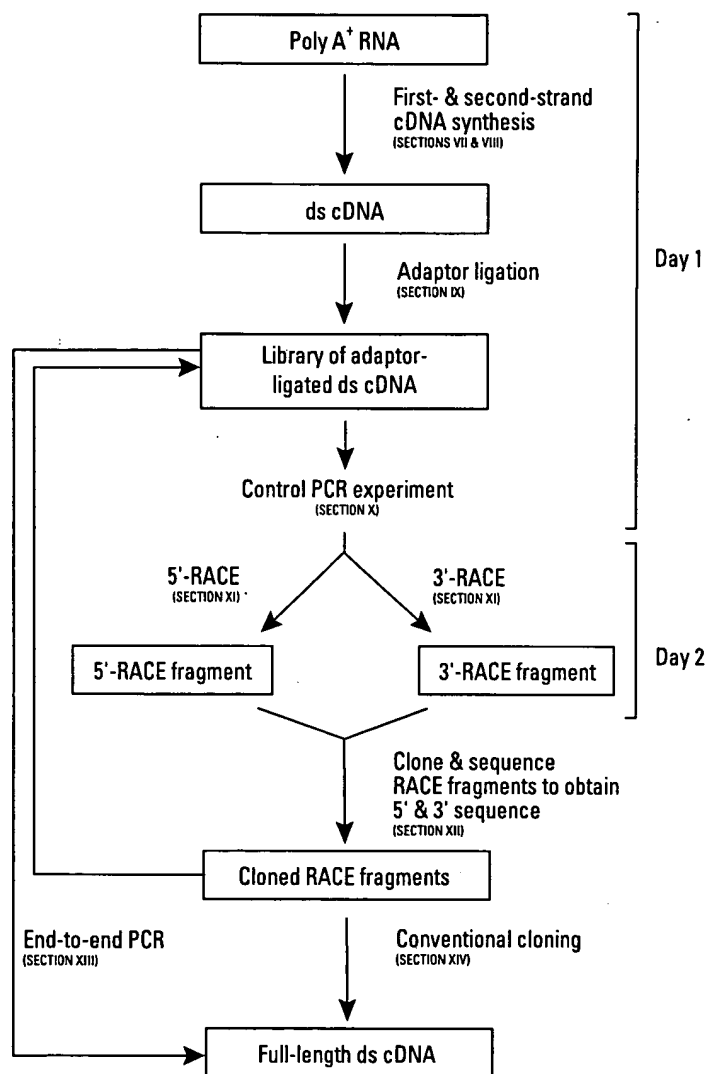


Figure 2. Overview of Marathon procedure. A more detailed flow chart of the Marathon procedure can be found in Appendix B. This figure depicts the order in which different products are generated. Note that with the cloned RACE fragments you can use a restriction site in an overlapping region and a site in the cloning vector to obtain both parts of the complete cDNA and subclone them together to obtain a full-length cDNA transcript or you can sequence the 5' end of the 5' product and the 3' end of the 3' product to obtain additional sequence information. Using this additional sequence information, you can design 5' and 3' gene-specific primers to use in LD PCR with the adaptor-ligated ds cDNA to obtain the full-length cDNA.

I. Introduction & Protocol Overview *continued*

Overview of the Marathon cDNA amplification protocol

An overview of Marathon cDNA amplification is presented in Figure 2. cDNA synthesis, adaptor ligation, and 5'- and/or 3'-RACE can be completed in two days. The time required to characterize the RACE products and to generate the full-length cDNA can vary greatly depending on the particular target. As you read the following description and set up your experiments, you may find it useful to refer to the detailed flow chart (Figure 9; Appendix B) and the diagram of the Marathon cDNA template and primers (Figure 4; Section V).

- **Primer Design (Section V)**

Gene-Specific Primers (GSPs) should be:

- 23–28 nt
- 50–70% GC
- $T_m \geq 65^\circ\text{C}$; best results are obtained if $T_m \geq 70^\circ\text{C}$ (enables the use of touchdown PCR)

You will need to design gene-specific primers for the 5'- and/or 3'-RACE reactions (GSP1 and GSP2, respectively). Nested primers (NGSP1 and NGSP2) will facilitate analysis of your RACE products, as described in Section XIII, and can be used for nested RACE PCR if necessary. Primer design is discussed in detail in Section V and Figure 4 shows the relationship of primers and template used in Marathon RACE reactions.

- **cDNA Synthesis (Sections VII & VIII)**

Marathon cDNA amplification begins with cDNA synthesis. We recommend starting with poly A⁺ RNA. We do not recommend using the Marathon Kit with total RNA or genomic DNA, because backgrounds tend to be higher and other researchers have reported difficulties when starting with these materials. (For applications using genomic DNA, we recommend using our GenomeWalker™ Kits.) First-strand synthesis uses a modified lock-docking oligo(dT) primer with two degenerate nucleotide positions at the 3' end. These nucleotides position the primer at the start of the poly-A tail and thus eliminate the 3' heterogeneity inherent with conventional oligo(dT) priming (Chenchik *et al.*, 1994; Borson *et al.*, 1994). The combination of reverse transcriptase (RT) and the carefully optimized Marathon reaction conditions give consistently high yields and size distributions of first-strand cDNA synthesis.

Second-strand synthesis is performed according to the method of Gubler & Hoffmann (1983) with a convenient cocktail of *E. coli* DNA polymerase I, RNase H, and *E. coli* DNA ligase. The conditions and enzyme concentrations for second-strand cDNA synthesis have been optimized to produce high yields of ds cDNA. Typically less than 15% of the second-strand syntheses primed by hairpin-loop formation.

I. Introduction & Protocol Overview *continued*

- **Adaptor Ligation (Section IX)**

Following creation of blunt ends with T4 DNA Polymerase, the ds cDNA is ligated to the Marathon cDNA Adaptor. (See Appendix A for information on the design and the sequence of the Marathon cDNA Adaptor.) This adaptor is partially double-stranded and is phosphorylated at the 5' end to facilitate blunt-end ligation of the adaptor to both ends of the ds cDNA by T4 DNA Ligase. Blunt-end ligation is more efficient than homopolymeric tailing or ligation of an adaptor to single-stranded cDNA by T4 RNA ligase, so a higher percentage of the resulting cDNA molecules contain the terminal structure required for RACE. This is a primary reason why Marathon 5'-RACE reactions are more efficient and reproducible than 5'-RACE methods based on tailing or single-stranded (ss) ligation (Frohman *et al.*, 1988; Dumas *et al.*, 1991; Harvey and Darlison, 1991). Prior to RACE, the adaptor-ligated cDNA is then diluted to a concentration suitable for Marathon RACE reactions.

- **Control RACE Experiment (Section X)**

Prior to performing RACE with your template, we *strongly* recommend that you perform a positive control RACE experiment with your 50X polymerase mix and the PCR Control cDNA provided with the kit.

- **Marathon RACE Reactions (Section XI)**

At this point, you essentially will have an uncloned library of adaptor-ligated ds cDNA. Furthermore, you will have enough material to perform 5'- and 3' Marathon RACE with many different genes simply by using different gene-specific primers.

Marathon RACE reactions should be performed with Advantage® 2 Polymerase Mix (#8430-1). Advantage 2 is a robust enzyme mix that is ideally suited for LD PCR and has been thoroughly tested with the protocols in this User Manual. This 50X mix contains TITANIUM™ Taq DNA Polymerase—a nuclease-deficient N-terminal deletion of Taq DNA polymerase plus TaqStart™ Antibody to provide automatic hot-start PCR (Kellogg *et al.*, 1994)—and a minor amount of a proofreading polymerase. Advantage 2 Polymerase Mix is also available in the Advantage® 2 PCR Kit (#K1905-1, -y).

If your cDNA of interest has high GC content, consider using Advantage-GC cDNA Polymerase Mix (#8419-1) or Kit (#K1907-1, -y). For applications in which the highest fidelity product is desired and templates are less than 2.5-kb long, use the Advantage-HF Polymerase Mix (#8419-1) or Kit (#K1909-1, -y). For more information, see Section XVI (Troubleshooting RACE Reactions).

If you choose not to use Advantage 2 Polymerase Mix, Table VI in Appendix C gives recommendations for mixing TaqStart Antibody (#5400-1) with other 50X LD PCR polymerase mixes.

I. Introduction & Protocol Overview *continued*

The ability to perform 5'- and 3'-RACE from the same template is made possible by the design of the Marathon cDNA Adaptor, which is described in detail in Appendix A. Both 5'- and 3'-RACE PCR reactions are primed with an internal gene-specific primer (GSP) and the Marathon Adaptor Primer (AP1). The adaptor-ligated cDNA does not contain a binding site for AP1. During the first round of thermal cycling, the GSP is extended to the end of the adaptor, creating an AP1 binding site at the 5' (or 3') terminus of the cDNA. In subsequent cycles, both AP1 and the GSP can bind, allowing exponential amplification of the cDNA of interest. Nonspecific products are greatly reduced because the AP1 binding cannot be created on the general population of cDNA molecules, which also lack binding sites for the GSPs.

- **Characterization of RACE Products (Section XII)**

Before generating the full-length cDNA, we strongly recommend that you characterize your RACE products to confirm that you have amplified the desired target. This can be done by one or more of the following: (1) comparing PCR products obtained using GSP1 and AP1 to product generated with NGSP1 and AP1; (2) probing a Southern blot of your PCR products with an internal gene-specific probe (e.g., labeled NGSP1); and (3) cloning and sequencing your RACE products. In general, we recommend that you obtain at least some sequence information.

Careful characterization of your RACE products at this point can prevent confusion and wasted effort in your subsequent experiments, even when both RACE reactions produce single major products. For example, when we cloned and sequenced the "single" RACE product observed with actin primers in Figure 1, we found that this single band actually contained cDNAs from three different actin genes. Characterization is essential at this point if you have multiple RACE products or suspect that you are working with a member of a multigene family.

- **Options for Generating Full-Length cDNA**

After RACE products have been characterized by partial or complete sequencing, the full-length cDNA can be generated by one of two methods:

- (1) **Generation of Full-Length cDNA by PCR (Section XIII)**

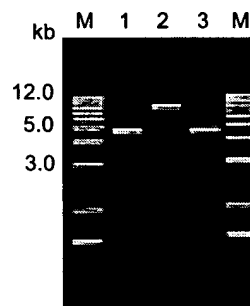
A standard LD-PCR reaction with GSPs from the 5' and 3'-ends of your gene can be used to amplify the full-length cDNA from the adaptor-ligated cDNA (see Figure 3, Section V.). The sequence of the 5' and 3' GSP is usually obtained by sequencing the 5' end of the 5'-RACE product and the 3' end of the 3'-RACE product, as described in Section XIII. Figure shows three examples of full-length cDNAs generated by end-to-end PCF

- (2) **Generation of Full-Length cDNA by Cloning (Section XIV)**

Cloned, overlapping 5'- and 3'- RACE fragments can be used to generate the full-length cDNA using a restriction site in the overlapping region (if one exists) and sites in the Marathon Adaptor and/or cDNA Synthesis Prime

I. Introduction & Protocol Overview *continued*

Figure 3. Examples of large full-length cDNAs generated by end-to-end PCR of Marathon-Ready cDNAs. Full-length cDNAs were generated using Advantage 2 Polymerase Mix and 5' and 3' GSPs obtained by partial sequencing of 5' and 3' Marathon RACE products. The template for Lanes 1 & 2 was Human Skeletal Muscle Marathon-Ready cDNA (#7413-1); the template for Lane 3 was Human Placenta Marathon-Ready cDNA (#7411-1). Lane 1: Full-length ILGFR1 cDNA (5.0 kb; 32 cycles; 7-min extension). Lane 2: Full-length ILGFR2 (8.9 kb; 28 cycles; 10-min extension). Lane 3: Full-length TFR cDNA (5.0 kb; 25 cycles; 7-min extension). M: 1-kb DNA ladder.



In general, PCR using flanking GSPs is more direct and less subject to complications or artifacts. With cloning, there is a slight chance of joining 5' and 3' cDNA fragments derived from two different transcripts; this could occur with two different forms of a polymorphic RNA or with transcripts from a multigene family. In contrast, with end-to-end PCR, the 5' and 3' GSPs will amplify the full length of a single cDNA, so there is no chance of generating a hybrid cDNA. Virtually all cDNAs are within the range of LD PCR.

No method of cDNA synthesis can guarantee a full-length cDNA, particularly at the 5' end. Determining the true 5' end requires some combination of RNase protection assays, primer extension assays, and cDNA or genomic sequence information. Many Marathon cDNAs include the complete 5' end of the cDNA; however, the action of T4 DNA polymerase may remove some nucleotides (typically 0–20) from the 5' end of the cDNA. Severe secondary structure may also block the action of RT and/or *Taq* DNA polymerase in some instances. In our experience, Marathon RACE products and full-length cDNAs compare favorably in this regard with cDNAs obtained by conventional RACE or from libraries. To obtain the maximum possible amount of 5' sequence, we recommend that you sequence the 5' end of 5–10 separate clones of the 5'-RACE product.

II. List of Components

Store poly A⁺ RNA at –70° C. Store DNA purification reagents at 4°C. Store all other reagents at –20°C.

The following reagents are sufficient for 5 cDNA synthesis reactions, 10 adaptor ligation reactions, and 100 PCR reactions.

Note: See Figure 8 (Appendix A) for the sequences of the Marathon cDNA Adaptor and Primers.

First-Strand Synthesis

- 5 µl AMV Reverse Transcriptase (20 units/µl)
- 10 µl cDNA Synthesis Primer (CDS Primer; 10 µM)
- 500 µl 5X First-Strand Buffer:
 - 250 mM Tris (pH 8.5)
 - 40 mM MgCl₂
 - 150 mM KCl
 - 5 mM Dithiothreitol (DTT)

Second-Strand Synthesis

- 25 µl 20X Second-Strand Enzyme Cocktail (*E. coli* DNA polymerase I [6 units/µl], *E. coli* DNA ligase [1.2 units/µl] and *E. coli* RNase H [0.25 units/µl])
- 500 µl 5X Second-Strand Buffer
 - 500 mM KCl
 - 50 mM Ammonium Sulfate
 - 25 mM MgCl₂
 - 0.75 mM β-NAD
 - 100 mM Tris (pH 7.5)
 - 0.25 mg/ml Bovine Serum Albumin
- 10 µl T4 DNA Polymerase (5 units/µl)

Adaptor Ligation

- 10 µl T4 DNA Ligase (400 units/µl)
- 20 µl Marathon cDNA Adaptor (10 µM)
- 500 µl 5X DNA Ligation Buffer
 - 250 mM Tris-HCl (pH 7.8)
 - 50 mM MgCl₂
 - 5 mM DTT
 - 5 mM ATP
 - 25% (w/v) Polyethylene glycol (MW 8,000)

II. List of Components *continued*

5'- & 3'-RACE PCR

- 100 µl Adaptor Primer 1 (AP1; 10 µM)
- 50 µl Nested Adaptor Primer 2 (AP2; 10 µM)

Control Reagents

- 5 µl Control Human Placental Poly A⁺ RNA (1 mg/ml)
- 75 µl PCR Control cDNA (ds cDNA made from Human Placental Poly A⁺ RNA and ligated to the Marathon Adaptor; ~0.1 µg/ml)
- 25 µl Control 5'-RACE TFR Primer (10 µM)
- 25 µl Control 3'-RACE TFR Primer (10 µM)

General Reagents

- 120 µl dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10 mM)
- 5 ml Tricine-EDTA Buffer
 - 10 mM Tricine-KOH (pH 8.5)
 - 0.1 mM EDTA
- 100 µl 20X EDTA/Glycogen Mix
 - 0.2 M EDTA
 - 2 mg/ml Glycogen
- 500 µl Ammonium Acetate (4 M)
- 5 ml Sterile Deionized H₂O

NucleoTrap Gel Extraction Kit (#K3070-1)

- 100 µl NucleoTrap Suspension
- 3 ml NT1 Buffer
- 10 ml NT2 Buffer
- 2 ml NT3 Buffer

III. Additional Materials Required

The following reagents are required but not supplied:

- **Advantage® 2 Polymerase Mix (50X)**

Marathon RACE reactions must be performed with a polymerase mix suitable for LD PCR; a single polymerase will *not* give satisfactory results in most experiments. We strongly recommend CLONTECH's Advantage 2 Polymerase Mix (#8430-1), as every lot is specifically tested in the Marathon RACE procedure. Advantage 2 contains TITANIUM™ *Taq* DNA Polymerase—a nuclease-deficient N-terminal deletion of *Taq* DNA polymerase plus TaqStart™ Antibody to provide automatic hot-start PCR (Kellogg *et al.*, 1994)—and a minor amount of a proofreading polymerase. Advantage 2 Polymerase Mix is also available in the Advantage 2 PCR Kit (#K1910-1, -y).

If you choose not to use Advantage 2 Polymerase Mix, we recommend that you use a 50X polymerase mix with the primary enzyme being *Taq* DNA polymerase or a *Taq* derivative. We do not recommend using *Tth* DNA polymerase. In our experience, Marathon RACE reactions tend to be less efficient and backgrounds tend to be higher with mixes containing *Tth*.

- **10X PCR reaction buffer** (Included with Advantage 2 Polymerase Mix.)
Use the 10X reaction buffer supplied with your source of native or truncated *Taq* DNA polymerase in all reactions that call for 10X PCR buffer.
- **0.5-ml PCR reaction tubes.** We recommend Perkin-Elmer GeneAmp 0.5-ml reaction tubes (cat. #N801-0737 or N801-0180).
- **80% Ethanol**
- **Phenol:chloroform:isoamyl alcohol (25:24:1)**
Melt phenol.
Equilibrate with an equal volume of sterile TNE buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA).
Incubate the mixture at room temperature for 2–3 hr.
Remove and discard the top layer.
Add an equal volume of chloroform:isoamyl alcohol (24:1) to the remaining layer and β-mercaptoethanol to 0.1 M. Mix thoroughly.
Remove and discard the top layer.
Store the bottom layer of phenol:chloroform:isoamyl alcohol at –20°C away from light. Do not store for more than two months.

III. Additional Materials Required *continued*

- Chloroform:isoamyl alcohol (24:1)
- 95% Ethanol
- [α -³²P]dCTP
We recommend that you add [α -³²P]dCTP to the first-strand synthesis reaction in order to quantify the cDNA yield and to determine the efficiency of ethanol precipitation. It is easier to troubleshoot the cDNA synthesis reaction if [α -³²P]dCTP is incorporated into the cDNA.

IV. General Considerations of Marathon cDNA Amplification

- The cycling parameters throughout this protocol have been optimized using a Perkin-Elmer DNA Thermal Cycler 480 or GeneAmp PCR Systems 2400/9600, the Advantage 2 PCR Kit, and the reagents and TFR controls provided in the Marathon Kit. The optimal cycling parameters may vary with different 50X polymerase mixes, templates, gene-specific primers, and thermal cyclers. Prior to performing 5'- and 3'-RACE with your experimental sample, it is important to perform the control PCR experiment (Section X) using the PCR Control cDNA and the Control 5'- and 3'- RACE TFR Primers, together with your 50X polymerase mix and DNA thermal cycler.

Please note that the efficiency of RACE PCR also depends on the abundance of the mRNA of interest in the poly A⁺ RNA sample and different primers will have different optimal annealing/extension temperatures. Refer to Section XVII for suggestions on optimizing PCR conditions.

- **You must use some form of hot start in the 5'-RACE and 3'-RACE PCR.** The following protocols have been optimized using TaqStart Antibody (Kellogg *et al.*, 1994) in the 50X polymerase mix. Hot start can also be performed using wax beads (Chou *et al.*, 1992) or manually (D'Aquila *et al.*, 1991).
- We recommend the Tricine-EDTA Buffer provided in the kit for resuspending and diluting your DNA samples throughout this protocol. Tricine buffers maintain their pH at high temperature better than Tris-based buffers. Tris-based buffers can lead to low pH conditions that can degrade DNA, particularly during the heat denaturation of the adaptor-ligated ds cDNA prior to RACE.
- Wear gloves throughout to protect your RNA samples from nucleases.
- When resuspending pellets or mixing reactions, gently pipet the solution up and down or tap the bottom of the tube, then spin briefly to bring all contents to the bottom of the tube.
- Vortex phenol:chloroform extractions.
- Perform all reactions on ice unless otherwise indicated.
- Add enzymes to reaction mixtures last. Make sure that the enzyme is thoroughly mixed with the reaction mixture by gently pipetting the mixture up and down.
- Use the recommended amounts of enzyme. These amounts have been carefully optimized for the Marathon amplification protocol and reagents.
- Ethidium bromide is a carcinogen. Use appropriate precautions in handling and disposing of this reagent. For more information, see *Molecular Cloning: A Laboratory Manual* by Sambrook *et al.* (1989).

V. Primer Design

A. Primer Sequence

Gene-Specific Primers (GSPs) should be:

- 23–28 nt
- 50–70% GC
- $T_m \geq 65^\circ\text{C}$; best results are obtained if $T_m \geq 70^\circ\text{C}$ (enables the use of touchdown PCR)

The relationship of the primers used in the Marathon RACE reactions to the template and resulting RACE products is shown in detail in Figure 4. For the complete Marathon protocol, you will need at least two GSPs: an antisense primer for the 5'-RACE PCR and a sense primer for the 3'-RACE PCR. If you are doing only 5'- or 3'-RACE, you will only need one GSP. All primers should be 23–28 nt long; there is generally no advantage to using primers longer than 30 nt. The primers shown in Figure 4 will create overlapping 5'- and 3'-RACE products, which, if a suitable restriction site is located in the region of overlap, can subsequently be joined by restriction digestion and ligation to create the full-length cDNA. Using primers designed to give overlapping RACE products also means that these primers can be used together to generate the overlapping fragment if the overlap is at least 100–200 bp. This provides a useful control for PCR. However, it is not absolutely necessary to use primers that give overlapping fragments. In the case of large and/or rare cDNAs, it may be better to use primers that are closer to the ends of the cDNA and therefore do not create overlapping fragments. Additionally, the primers themselves can overlap (i.e., be complementary).

GSPs should have GC content of 50–70% and a T_m of at least 65°C ; whenever possible the T_m should be 70°C or higher as determined by nearest neighbor analysis (Freier *et al.* [1986]). In our experience, longer primers with annealing temperatures of at least 70°C give more robust amplification in RACE, particularly from difficult samples. T_m 's of 70°C or higher allow you to use "touchdown PCR" (Section C below). T_m 's of GSP1 and GSP2 can be calculated or determined experimentally by doing PCR at different temperatures. Avoid using self-complementary primer sequences which can fold back and form intramolecular hydrogen bonds. Similarly, avoid using primers that have complementarity to the Marathon AP1 Primer, particularly in the 3' ends.

Note: Do not incorporate restriction sites into the 5' ends of the 5' and 3' GSPs. In our experience, the presence of these extra sequences can lead to increased backgrounds.

V. Primer Design *continued*

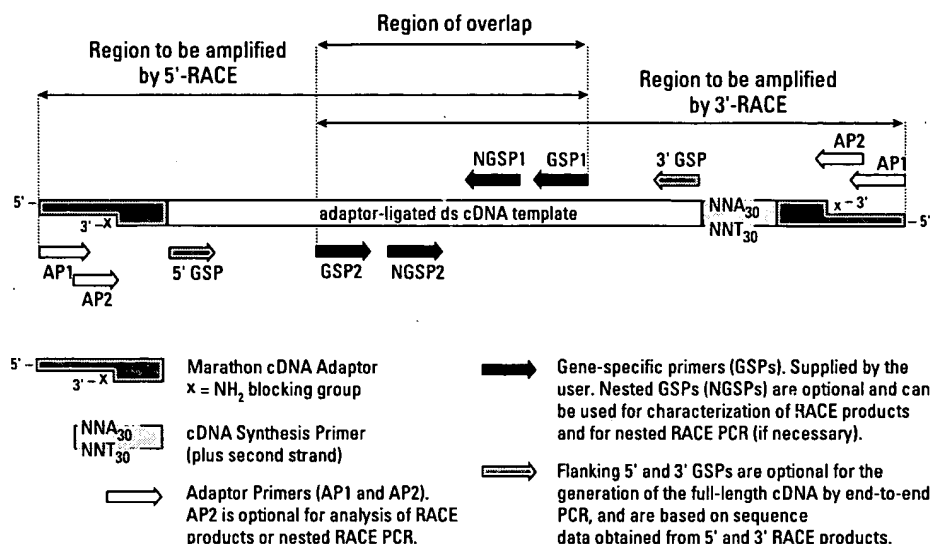


Figure 4. The template and primers used in Marathon RACE reactions. cDNA synthesis and adaptor ligation create a population of cDNAs with the structure depicted above. This population is essentially a library of uncloned ds cDNA from which you can amplify many different cDNAs using different sets of GSPs. If the GSPs create overlapping 5'- and 3'-RACE products, the products can subsequently be joined by restriction digestion and ligation to create the full-length cDNA (provided there are suitable restriction sites in the region of overlap). Also, GSPs designed to give overlapping RACE products can be used together to generate a useful control for PCR. However, it is not absolutely necessary to use primers that give overlapping fragments. The specificity of Marathon RACE reactions is greatly enhanced by the absence of an AP1 binding site on the adaptor-ligated cDNAs. This site is created on the cDNA of interest by extension from the GSP during the first RACE cycle. The amine group on the Marathon cDNA Adaptor blocks extension of the 3' end of the adaptor-ligated ds cDNAs, and thus prevents formation of an AP1 binding site on the general population of cDNAs.

B. Location of Primer Sequences within Gene

We have had good success using the Marathon Kit to amplify 5' and 3' cDNA fragments that extend up to 6.5 kb from the GSP sites. If possible, choose your primers so that the 5'- and 3'-RACE products will be 3 kb or less.

If designing primers that produce overlapping 5'- and 3'-RACE products, it is helpful to design the gene-specific PCR primers so that the overlap between GSP1 and GSP2 is at least 100–200 bases. In this way, a stretch of known sequence will be incorporated into the amplified 5' and 3' fragments and can be used to verify that the correct gene was amplified.

V. Primer Design *continued*

C. Touchdown PCR

We have found that touchdown PCR (Don *et al.*, 1991; Roux, 1995) significantly improves the specificity of Marathon RACE PCR. Touchdown PCR involves using an annealing/extension temperature, during the initial PCR cycles, that is several degrees *higher* than the T_m of the AP1 Primer. (T_m of AP1 = 71°C.) If the T_m of your GSP >70°C, *only* gene-specific synthesis occurs during these initial cycles, and this allows a critical amount of gene-specific product to accumulate. The annealing/extension temperature is then reduced to the AP1 Primer T_m for the remaining PCR cycles, permitting efficient, exponential amplification of the gene-specific template.

As noted above, we recommend using primers with T_m 's >70°C to allow you to use the touchdown cycling programs in the protocol. (Nontouchdown cycling programs are also included for use with primers with T_m 's <70°C.)

D. Nested Primers

We recommend that you do *not* use nested PCR in your initial experiments. The AP1 Primer and a GSP will usually generate a good RACE product with a low level of nonspecific background. However, a Southern blot or nested GSPs (NGSP1 and NGFP2; see Figure 4) are very useful for characterizing your RACE products. Furthermore, nested PCR may be necessary in some cases where the level of background or nonspecific amplification in the 5'- or 3'-RACE reaction is too high with a single GSP. In nested PCR, a primary amplification is performed with the outer primers and, if a smear appears, then an aliquot of the primary PCR product is reamplified using the inner primers. The Marathon protocols include optional steps indicating where nested primers can be used. The nested AP2 Primer provided with the kit can be used for both 5'- and 3'-RACE.

Nested primers should be designed according to the guidelines discussed above. If possible, nested primers should not overlap (like AP1 and AP2); if they must overlap (due to limited sequence information), the 3' end of the inner primer should have as much unique sequence as possible. Be sure that nested primers do not contain sequences that can hybridize to the outer gene-specific primer, particularly at their 3' ends.

E. Controls to Test Gene-Specific Primers (GSPs)

When performing the RACE reactions, we recommend that you perform the following controls to test your GSPs:

1. Negative control with single primers

Include a negative control containing only the appropriate GSP (antisense primer for 5'-RACE; sense primer for 3'-RACE) and the adaptor-ligated ds cDNA. The GSPs should not give any bands in the

V. Primer Design *continued*

absence of the AP1 Primer. If significant amounts of product are seen with this control, it may be necessary to alter the cycling parameters, use nested primers, or redesign your original primer.

2. Positive control with both GSPs (Only possible if using primers that produce overlapping 5' and 3' fragments.)

To confirm that your gene is expressed in your RNA sample, set up a positive control containing both GSPs and your adaptor-ligated ds cDNA. This should produce a band corresponding to the combined length of your GSPs and the overlap between the primers (i.e., the region of overlap between the 5'- and 3'-RACE products). If this band is missing, it may be necessary to repeat your cDNA synthesis or to start with a different tissue/cell source.

VI. Preparation & Handling of Poly A⁺ RNA

A. General Precautions

The integrity and purity of your poly A⁺ RNA used as starting material is an important element of high-quality cDNA synthesis. The following precautions will help you avoid contamination and degradation of your RNA:

- Wear gloves.
- Use freshly deionized (e.g., MilliQ-grade) H₂O directly, without treatment with DEPC (diethyl pyrocarbonate).
- Rinse all glassware with 0.5 N NaOH, followed by deionized H₂O. Then bake the glassware at 160–180°C for 4–9 hr.
- Use only single-use plastic pipettes and pipette tips.

B. RNA Isolation

Many procedures are available for the isolation of poly A⁺ RNA (Farrell, 1993; Sambrook *et al.*, 1989).

C. RNA Analysis

After isolating poly A⁺ RNA, we recommend that you examine it by electrophoresis of a sample on a denaturing formaldehyde agarose/EtBr gel. Poly A⁺ RNA samples from mammalian cells should produce smears from 0.5–12 kb with much weaker ribosomal RNA bands at approximately 4.5 and 1.9 kb. Size distribution may be smaller with nonmammalian tissue sources.

VII. First-Strand cDNA Synthesis

The 10- μ l reaction described below converts 1 μ g of poly A⁺ RNA into first-strand cDNA. Following second-strand synthesis (Section VIII), the ds cDNA is ready for ligation to the Marathon cDNA Adaptor (Section IX). This kit contains enough components for five separate cDNA syntheses.

We strongly recommend that you perform a positive control cDNA synthesis using the Human Placental Poly A⁺ RNA that is included in the kit. This will verify that the system performs in your hands and will allow you to estimate the yield and size distribution of the ds cDNA synthesized from your RNA. This information is very important for successful amplification of full-sized 5' & 3' cDNA fragments. The ds cDNA you make from the positive control RNA will—together with the TFR Primers—provide a positive control for the 5'- and 3'-RACE PCR reactions described in Sections XI and XII.

1. Combine the following in a sterile 0.5-ml microcentrifuge tube:

1 μ g (1–4 μ l)	RNA sample (poly A ⁺)
1 μ l	cDNA Synthesis Primer (10 μ M)

2. Add sterile H₂O to a final volume of 5 μ l.
3. Mix contents and spin the tube briefly in a microcentrifuge.
4. Incubate the tube at 70°C for 2 min.
5. Cool the tube on ice for 2 min.
6. Spin the tube briefly to collect the contents at the bottom.
7. Add the following to each reaction tube:

2 μ l	5X First-Strand Buffer
1 μ l	dNTP Mix (10 mM)
1 μ l	[α - ³² P]dCTP (1 μ Ci/ μ l) *
1 μ l	AMV Reverse Transcriptase (20 units/ μ l)
10 μ l	Total volume

* [α -³²P]dCTP is optional. Addition of the isotope facilitates tracking your cDNA through subsequent steps and troubleshooting cDNA synthesis. If you choose not to add [α -³²P]dCTP, add 1 μ l of H₂O. If using [α -³²P]dCTP with a specific activity of 10 μ Ci/ μ l, be sure to dilute 10-fold with H₂O.

8. Mix the contents of the tube by gently pipetting.
9. Spin the tube briefly to collect the contents at the bottom.
10. Incubate the tube at 42°C for 1 hr in an air incubator.

Note: Using a water bath or thermalcycler for this incubation may reduce the volume of the reaction mixture (due to evaporation) and therefore reduce the efficiency of first-strand synthesis.

11. Place the tube on ice to terminate first-strand synthesis.
12. Proceed directly to second-strand synthesis.

VIII. Second-Strand cDNA Synthesis

This procedure describes an 80- μ l reaction in which second-strand cDNA is synthesized from the first-strand cDNA produced in the 10- μ l first-strand reaction. The Second-Strand Enzyme Cocktail contains RNase H, *E. coli* DNA polymerase I, and *E. coli* DNA ligase. These enzymes degrade the RNA and synthesize the second cDNA strand. The action of T4 DNA Polymerase in Steps 4–5 creates blunt ends on the ds cDNA.

We recommend that you also perform a positive control second-strand synthesis using the first-strand cDNA made from the Human Placental Poly A⁺ RNA provided with the kit. This is necessary if you want to have a positive control for later steps.

Note: All components and reaction vessels should be prechilled on ice.

- Combine the following components in the reaction tube from Step VII.11:

(10 μ l	First-strand reaction)
48.4 μ l	Sterile H ₂ O
16 μ l	5X Second-Strand Buffer
1.6 μ l	dNTP Mix (10 mM)
4 μ l	20X Second-Strand Enzyme Cocktail
80 μ l	Total volume

- Mix contents thoroughly with gentle pipetting.
- Spin the tube briefly to collect the contents at the bottom.
- Incubate the tube at 16°C for 1.5 hr.
- Add 2 μ l (10 units) of T4 DNA Polymerase and mix thoroughly with gentle pipetting.
- Incubate the tube at 16°C for 45 min.
- Add 4 μ l of the EDTA/Glycogen Mix to terminate second-strand synthesis.
- Add 100 μ l of phenol:chloroform:isoamyl alcohol (25:24:1).
- Vortex thoroughly.
- Spin the tube in a microcentrifuge at 14,000 rpm for 10 min to separate phases.
- Carefully transfer the top aqueous layer to a clean 0.5-ml microcentrifuge tube. Discard the interface and lower phase.
- Add 100 μ l of chloroform:isoamyl alcohol (24:1) to the aqueous layer and vortex thoroughly.
- Spin the tube in a microcentrifuge at 14,000 rpm for 10 min to separate phases.
- Remove the top aqueous layer and place in a clean 0.5-ml microcentrifuge tube.

VIII. Second-Strand cDNA Synthesis *continued*

15. Add one-half volume of 4 M Ammonium Acetate. (e.g., if you recovered ~70 µl at Step 14, add 35 µl of 4 M Ammonium Acetate.)
16. Add 2.5 volumes of room-temperature 95% ethanol. (e.g., if your volume at Step 15 was ~105 µl, add 263 µl of 95% ethanol.)
17. Vortex the mixture thoroughly.
18. Spin the tube immediately in a microcentrifuge at 14,000 rpm at room temperature for 20 min.

Note: Do not chill the ethanol precipitate prior to centrifugation. Incubation at low temperatures does not improve the yield of ethanol precipitation with ammonium acetate and may precipitate impurities that will inhibit subsequent steps.
19. Remove the supernatant carefully.
(If you included [α -³²P]dCTP in the reaction, check the efficiency of the precipitation. The pellet should contain 1–10% of the total radioactivity in the sample.)
20. Gently overlay the pellet with 300 µl of 80% ethanol.
21. Spin in a microcentrifuge at 14,000 rpm for 10 min.
22. Carefully remove the supernatant.
(If you included [α -³²P]dCTP, check that the pellet still contains counts to be sure that you did not lose the sample during washing.)
23. Air dry the pellet for approximately 10 min to evaporate residual ethanol.
24. Dissolve the precipitate in 10 µl of H₂O and store at –20°C.
25. [Optional] We recommend that you estimate the yield of your experimental ds cDNA products and compare the yield and size range to the ds cDNA made from the positive control Human Placental RNA. This information will help you dilute your sample in Step IX.5.
 - a. Analyze 2 µl of your experimental ds cDNA and 2 µl of the positive control ds cDNA on a 1.2% agarose/EtBr gel with suitable DNA size markers.
 - b. If you cannot see your experimental ds cDNA via EtBr staining and you included [α -³²P]dCTP, dry the agarose gel using a vacuum gel drying system and expose x-ray film to the gel overnight at –70°C.

VIII. Second-Strand cDNA Synthesis *continued*

The yield of ds cDNA for experimental RNAs will depend on the quality of the poly A⁺ RNA. 1 µg of the Control Human Placental Poly A⁺ RNA will typically produce about 1 µg of ds cDNA. Similar amounts (0.5–1.0 µg) are typically obtained from high-quality experimental poly A⁺ RNAs.

Figure 5 shows two examples of ds cDNA produced from human poly A⁺ RNA. ds cDNA derived from poly A⁺ RNA typically appears as a smear from 0.5–10 kb on an agarose/EtBr gel. Bright bands corresponding to abundant mRNAs (or rRNAs) are common, as seen in Figure 5. (Size distribution may be smaller [0.5–3 kb] for some RNA samples from nonmammalian species.)

The following steps will also help you troubleshoot the reaction if yields are suboptimal:

1. Perform first- and second-strand cDNA synthesis with both your experimental sample and the Control Human Placental Poly A⁺ RNA provided in the kit.
2. Monitor synthesis and purification of the cDNA by including [α -³²P]dCTP in the first-strand reaction mixture.

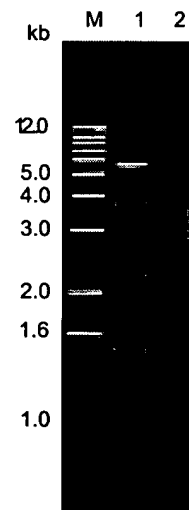


Figure 5. Typical results of ds cDNA synthesis. ds cDNA was prepared according to Sections VII & VIII using poly A⁺ RNA from skeletal muscle (Lane 1) or human placenta (Lane 2). 2 µl (of 10 µl total) was loaded in each lane. Lane M: 1-kb DNA size markers.

IX. Adaptor Ligation

The procedure below describes a 10- μ l reaction in which the Marathon cDNA Adaptor is ligated to the ds cDNA obtained after second-strand synthesis. Note that this reaction uses only half of the ds cDNA produced in Section VIII. This kit contains enough components for 10 separate ligations of the Marathon cDNA Adaptor to ds cDNA.

We recommend that you perform a positive control adaptor ligation using the ds cDNA made from the Human Placental Poly A⁺ RNA provided with the kit. This is necessary if you want to have a positive control for later steps.

Note: Allow 5X DNA Ligation Buffer to completely thaw at room temperature and keep it at room temperature for 30 min before use. **Do not put the 5X DNA Ligation Buffer on ice.**

1. Combine the following reagents in a 0.5-ml microcentrifuge test tube at room temperature and in the order shown:

5 μ l	ds cDNA
2 μ l	Marathon cDNA Adaptor (10 μ M)
2 μ l	5X DNA Ligation Buffer
1 μ l	T4 DNA Ligase (400 units/ μ l)
<hr/>	
10 μ l	Total volume
2. Mix by vortexing and spin briefly in a microcentrifuge.
3. Incubate at either:
 - 16°C overnight; or
 - room temperature (19–23°C) for 3–4 hr.
4. Heat at 70°C for 5 min to inactivate the ligase.
5. Using the following guidelines, dilute your adaptor-ligated ds cDNA to a concentration which is suitable for subsequent RACE PCR procedures (~0.1 μ g/ml).
 - a. If you compared your yield of ds cDNA to that obtained with the positive control RNA:
 - If the yield of your experimental sample is equal to or greater than the yield of the positive control, dilute 1 μ l of the reaction mixture with 250 μ l of Tricine-EDTA Buffer.
 - If the yield of your experimental sample is less than the yield of the positive control, dilute the reaction mixture with proportionately less Tricine-EDTA Buffer. For example, if your sample contained 5-fold less cDNA than the positive control, dilute 1 μ l in 50 μ l of Tricine-EDTA Buffer.

(If you cannot see your ds cDNA with EtBr staining, you will probably need to repeat Sections VII–IX using fresh, or more, poly A⁺ RNA.)

IX. Adaptor Ligation *continued*

- b. If you did not compare your yield of ds cDNA to that obtained with the positive control RNA, prepare separate 1/50 and 1/250 dilutions of adaptor-ligated ds-cDNA in Tricine-EDTA Buffer. Perform the subsequent RACE PCR reactions using the specified amount of both dilutions until you determine which dilution gives you the best results.
6. Dilute 1 µl of the positive control reaction mixture with 250 µl of Tricine-EDTA Buffer.
7. Store the undiluted adaptor-ligated cDNA at -20°C for future use.
(If your RACE reactions generate smears, you may wish to prepare a more dilute sample of the adaptor-ligated ds cDNA. For more information, please refer to Section XVII.)
8. Heat the diluted ds cDNA at 94°C for 2 min to denature the ds cDNA.
9. Cool the tube on ice for 2 min.
10. Briefly spin the tube in a microcentrifuge to collect the contents in the bottom of the tube.

Store at -20°C until ready for RACE PCR.

At this stage, you essentially have a library of adaptor-ligated ds cDNA. The RACE reactions in Sections XI and XII use only a fraction of this material for each RNA of interest. There is enough material to perform the rest of the protocol using GSPs for several different RNAs. This kit contains enough primers and dNTPs to perform 100 standard 50-µl PCR reactions.

X. Control PCR Experiment

Prior to performing 5'- and 3'-RACE reactions with your adaptor-ligated ds cDNA, we *strongly* recommend that you perform the following positive control RACE PCR experiment. This can save you considerable time by making sure the Marathon RACE protocol works with your enzyme and thermal cycler. If problems arise later in the protocol, the results of this experiment will help you determine immediately if the problem is with your RACE PCR (e.g., inactive enzyme mix, different thermal cycler) or with your adaptor-ligated ds cDNA. *This control is essential if you are not using Advantage 2 Polymerase Mix and a Perkin-Elmer DNA Thermal Cycler 480 or GeneAmp Systems 2400/9600.* If you choose not to use Advantage 2 Polymerase Mix, you must use your polymerase mix with some form of hot start PCR (i.e., TaqStart Antibody, wax beads, or manual hot start) to minimize background in your RACE reactions. Please note that all Marathon PCR reactions have been optimized with Advantage 2.

1. Prepare enough master mix for all PCR reactions and 1 extra reaction to ensure sufficient volume. For each 50- μ l PCR reaction, mix the following reagents:
 - 36 μ l H₂O
 - 5 μ l 10X cDNA PCR Reaction Buffer
 - 1 μ l dNTP Mix (10 mM)
 - 1 μ l Advantage 2 Polymerase Mix (50X)
 - 43 μ l Final volume
2. Mix well by vortexing (without introducing bubbles), then briefly spin the tube in a microcentrifuge.
3. Prepare PCR reactions as shown in Table II. Add the components to 0.5-ml PCR tubes in the order shown and mix gently.

TABLE II: SETTING UP THE CONTROL RACE EXPERIMENT

Component	Tube #: Description:	1 5'-RACE Control	2 3'-RACE Control	3 Internal Control
PCR Control cDNA		5 μ l	5 μ l	5 μ l
5'-RACE TFR Primer (10 μ M)		1 μ l	---	1 μ l
3'-RACE TFR Primer (10 μ M)		---	1 μ l	1 μ l
AP1 Primer (10 μ M)		1 μ l	1 μ l	---
Master Mix		43 μ l	43 μ l	43 μ l
Total volume		50 μ l	50 μ l	50 μ l
Expected product size		2.6 kb	2.9 kb	0.3 kb

X. Control PCR Experiment *continued*

4. Overlay the contents of each tube with 2 drops of mineral oil and place caps firmly on each tube.

Note: This is not necessary if you are using a "hot-lid" thermal cycler.

5. Commence thermal cycling using the following program for touchdown PCR.

Perkin-Elmer (PE) DNA
Thermal Cycler 480:

- 94°C for 1 min
- 5 cycles:
 - 94°C 30 sec
 - 72°C 4 min
- 5 cycles:
 - 94°C 30 sec
 - 70°C 4 min
- 20–25 cycles:
 - 94°C 20 sec
 - 68°C 4 min

PE GeneAmp Systems 2400/9600
(or hot-lid thermal cycler):

- 94°C for 30 sec
- 5 cycles:
 - 94°C 5 sec
 - 72°C 4 min
- 5 cycles:
 - 94°C 5 sec
 - 70°C 4 min
- 20–25 cycles:
 - 94°C 5 sec
 - 68°C 4 min

Note: Cycling parameters for both touchdown and conventional PCR are provided in Sections XI & XII (5'- and 3'-RACE, respectively). Both sets of parameters should give good results in this control experiment. However, we recommend using touchdown PCR whenever possible to optimize your chances for success with your primers. Touchdown PCR requires that your GSPs have a $T_m \geq 70^\circ\text{C}$.

6. Analyze 5 μl of each sample on a 1.2 % agarose/EtBr gel. (Store the remaining 45 μl of each reaction at -20°C until you are sure the control experiment has worked.)

Expected results (see lanes 4 & 5 of Figure 1 in the Introduction): The 5' control reaction should produce a 2.6-kb band. The 3' control reaction should produce a 2.9-kb band. If you do not observe these bands, try cycling the remaining portion of the reaction for 5 additional cycles.

If you have to perform 35 cycles or more to see the desired product, or if you cannot get any product at all, there may be a problem with your 50X polymerase mix. Before you attempt 5'- and 3'-RACE with your primers and adaptor-ligated ds cDNA, repeat the positive control experiment after switching to Advantage 2 Polymerase Mix or preparing a fresh 50X polymerase mix as described in Table VI. Use this reaction to optimize the PCR cycling parameters in your hands. We recommend that you do not proceed until you get the positive control experiment to produce single strong bands of the desired size in 35 cycles (5 cycles at 72°C + 5 cycles at 70°C + 25 cycles at 68°C).

XI. Rapid Amplification of cDNA Ends (RACE)

The procedure below describes the RACE PCR reactions that generate the 5' and 3' cDNA fragments. *We strongly recommend that you perform all of the controls shown in Tables III (5' RACE) and IV (3' RACE).* The positive controls using the positive control primers and AP1 (Tube # 2 in Tables III and IV) are absolutely essential. Controls 3–5 will provide additional useful information if your initial RACE reactions do not give the expected results. Although a Nested Adaptor Primer (AP2) is provided, nested PCR is generally not necessary in Marathon RACE reactions unless indicated by the controls in your initial experiments. For a complete discussion of controls, see Section XVI (Troubleshooting RACE Reactions).

All Marathon RACE reactions have been optimized with CLONTECH's Advantage 2 Polymerase Mix, which includes TaqStart Antibody for automatic hot start PCR. If you choose not to use Advantage 2 Polymerase Mix, Table VI (Appendix C) provides recommendations on how to use other licensed LD PCR polymerase mixes. You must use some form of hot start PCR (i.e., TaqStart Antibody, wax beads, or manual hot start) to minimize background in your RACE reactions.

1. Prepare enough PCR master mix for all of the PCR reactions plus one additional tube. The same master mix can be used for both 5'- and 3'-RACE reactions. For each 50- μ l reaction, mix the following reagents:

36 μ l	H ₂ O
5 μ l	10X cDNA PCR Reaction Buffer
1 μ l	dNTP Mix (10 mM)
1 μ l	Advantage 2 Polymerase Mix (50X)
43 μ l	Final volume

Mix well by vortexing (without introducing bubbles) and briefly spin the tube in a microcentrifuge.

2. **For 5'-RACE:** prepare PCR reactions as shown in Table III.
For 3'-RACE: prepare PCR reactions as shown in Table IV.
Add the components in the order shown in 0.5-ml PCR tubes.

XI. Rapid Amplification of cDNA Ends (RACE) *continued*

TABLE III: SETTING UP 5'-RACE PCR REACTIONS

Component	Test Tube #: Description:	1		2		3		4		5	
		Experimental	Sample	TFR	Pos. Ctrl ^a	GSP 1+2	Pos. Ctrl ^b	AP1 only	Neg. Ctrl ^c	GSP1 only	Neg. Ctrl ^c
Diluted adaptor-ligated expt'l cDNA		5 µl		---		5 µl		5 µl		5 µl	
Diluted adaptor-ligated pos. ctrl cDNA		---		5 µl		---		---		---	
AP1 Primer (10 µM)		1 µl		1 µl		---		1 µl		---	
GSP1 (antisense primer; 10 µM)		1 µl		---		1 µl		---		1 µl	
GSP2 (sense primer; 10 µM)		---		---		1 µl		---		---	
Control 5'-RACE TFR Primer (10 µM)		---		1 µl		---		---		---	
H ₂ O		---		---		---		1 µl		1 µl	
Master Mix		43 µl		43 µl		43 µl		43 µl		43 µl	
Final volume		50 µl		50 µl		50 µl		50 µl		50 µl	

Notes

^a The TFR Positive Control should generate a 2.6-kb product.

^b If performing both 5'- and 3'-RACE with overlapping products, the "GSP 1+2" Positive Control is useful to confirm that the gene of interest is expressed in the RNA sample. This control can only be performed if your GSP1 & 2 primers are designed to give overlapping 5'- and 3'-RACE products. An alternative is to use the control 5'- and 3'-RACE TFR Primers with 5 µl of your positive control cDNA or the PCR Control cDNA provided with the kit. This will generate a 290-bp fragment.

^c The "AP1 only" and "GSP1 only" negative controls are particularly useful if your 5'-RACE reactions produce a smear or extra bands. If one of these controls also produces a smear or extra bands similar to what was observed in the experimental RACE reaction, you may need to design new primary primers or perform a secondary RACE amplification with the appropriate nested primer(s).

XI. Rapid Amplification of cDNA Ends (RACE) *continued*

TABLE IV: SETTING UP 3'-RACE PCR REACTIONS

Component	Test Tube #: Description:	1		2		3		4		5	
		Experimental	Sample	TFR	Pos. Ctrl ^a	GSP 1+2	Pos. Ctrl ^b	AP1 only	Neg. Ctrl ^c	GSP2 only	Neg. Ctrl ^c
Diluted adaptor-ligated expt'l cDNA		5 µl		---		5 µl		5 µl		5 µl	
Diluted adaptor-ligated pos. ctrl cDNA		---		5 µl		---		---		---	
AP1 Primer (10 µM)		1 µl		1 µl		---		1 µl		---	
GSP2 (sense primer; 10 µM)		1 µl		---		1 µl		---		1 µl	
GSP1 (antisense primer; 10 µM)		---		---		1 µl		---		---	
Control 3'-RACE TFR Primer (10 µM)		---		1 µl		---		---		---	
H ₂ O		---		---		---		1 µl		1 µl	
Master Mix		43 µl		43 µl		43 µl		43 µl		43 µl	
Final volume		50 µl		50 µl		50 µl		50 µl		50 µl	

Notes

^a The TFR Positive Control should generate a 2.9-kb product.

^b If performing both 5'- and 3'-RACE with overlapping products, the "GSP 1+2" positive control is a useful control to confirm that the gene of interest is expressed in the RNA sample. This control can only be performed if your GSP1 & 2 primers are designed to give overlapping products. An alternative is to use the control 5'- and 3'-RACE TFR Primers with 5 µl of your positive control cDNA or the PCR Control cDNA provided with the kit. This will generate a 290-bp fragment.

^c The "AP1 only" and "GSP2 only" negative controls are particularly useful if your 3'-RACE reactions produce a smear or extra bands. If one of these controls also produces a smear or extra bands similar to what was observed in the experimental RACE reaction, you may need to design new primary primers or perform a secondary RACE amplification with the appropriate nested primer(s).

XI. Rapid Amplification of cDNA Ends (RACE) *continued*

- Overlay the contents of each tube with 2 drops of mineral oil and place caps firmly on each tube.

Note: This is not necessary if you are using a hot-lid thermal cycler.

- Commence thermal cycling using one of the following programs (programs 1 and 2 work with the positive control 5'-RACE TFR and AP1 Primers):

Program 1 (preferred; use if GSP $T_m > 70^\circ\text{C}$):

PE DNA Thermal Cycler 480: PE GeneAmp Systems 2400/9600 (or hot-lid thermal cycler):

- | | |
|-------------------------|-------------------------|
| • 94°C for 1 min | • 94°C for 30 sec |
| • 5 cycles: | • 5 cycles: |
| 94°C 30 sec | 94°C 5 sec |
| 72°C 4 min [◊] | 72°C 4 min [◊] |
| • 5 cycles: | • 5 cycles: |
| 94°C 30 sec | 94°C 5 sec |
| 70°C 4 min [◊] | 70°C 4 min [◊] |
| • 20–25 cycles: | • 20–25 cycles: |
| 94°C 20 sec | 94°C 5 sec |
| 68°C 4 min [◊] | 68°C 4 min [◊] |

Program 2 (Recommended only if GSP $T_m = 60\text{--}65^\circ\text{C}$; however, customers occasionally report success with Program 2 after not succeeding with Program 1, especially with problematic primers.)

PE DNA Thermal Cycler 480: PE GeneAmp Systems 2400/9600 (or hot-lid thermal cycler):

- | | |
|-------------------------|-------------------------|
| • 94°C for 1 min | • 94°C for 30 sec |
| • 25–30 cycles: | • 25–30 cycles: |
| 94°C 30 sec | 94°C 5 sec |
| 68°C 4 min [◊] | 68°C 4 min [◊] |

Notes on cycling:

You may need to determine the optimal cycling parameters for your gene empirically. If you see weak bands or no bands, perform five additional cycles at 68°C. For more suggestions on optimizing RACE PCR conditions, refer to Section XVII.

[◊] The optimal extension time depends on the length of the fragment being amplified. We typically use 4 min for cDNA fragments of 2–5 kb. For 0.2–2-kb targets, we reduce the extension time to 2–3 min. For 5–10-kb targets, we increase the extension time up to 10 min.

- When cycling is completed, analyze 5 μl from each tube, along with appropriate DNA size markers, on a 1.2% agarose/EtBr gel.

Note: Figure 1 in the Introduction shows sample results.

XI. Rapid Amplification of cDNA Ends (RACE) *continued*

6. [Optional] If the primary PCR reaction fails to give the distinct band(s) of interest or produces a smear, you may wish to perform a Southern blot using:

- a. a cDNA probe
- b. a nested primer as a probe

Or, you may wish to perform a secondary, or "nested," PCR reaction using the AP2 primer supplied with Marathon-Ready cDNA and a NGSP. (See the discussion in Section V. Primer Design.)

- a. Dilute 5 μ l of the primary PCR product into 245 μ l of Tricine-EDTA buffer.
- b. Repeat steps 1–5 above, using:
 - 5 μ l of the diluted primary PCR product in place of the Marathon-Ready cDNA.
 - 1 μ l of the AP2 primer and 1 μ l of your nested antisense GSP.
 - Fewer cycles (15–20 instead of 25–30).

At CLONTECH, we have successfully used the Marathon Kit to amplify the 5'- and 3'-RACE fragments of several different genes from poly A⁺ RNA. Typically, only one major band is generated, although in some cases minor bands are also visible. Although provided in the kit, nested primers generally are *not* needed for successful Marathon amplification. If you do not know the complete structure of your gene, you may be able to predict the size of the correctly amplified product via Northern blot analysis. Certain genes will give multiple bands due to the presence of a multigene family or multiple RNAs. If there are multiple products, you may need to determine which are real (e.g., the products of alternative transcription start sites, alternative splicing sites, or related genes) and which are artifacts (e.g., the result of pausing by RT, high GC content, nonspecific priming during RACE PCR, etc.).

XII. Characterization of RACE Products

At this point, we recommend that you characterize your RACE fragments and confirm that you have amplified the desired product. This can prevent confusion and wasted effort when you try to generate the full-length cDNA, even if you have single major products from both the 5'- and 3'-RACE reactions. Characterization is especially important if you have multiple bands or if you suspect that you are working with a member of a multigene family. For example, the "single" RACE products generated using actin primers in Figure 1 (Introduction) contain cDNAs from three different members of the actin gene family.

Below we describe three methods for characterizing RACE products: (1) Comparison of RACE products obtained with GSPs and NGSPs; (2) Southern blotting; and (3) Cloning and sequencing. We recommend that you obtain at least some sequence confirmation before attempting to generate the full-length cDNA. For options 1 and 2, you will need nested GSPs for analyzing your 5'- and 3'-RACE products. Section XVII also contains information that may help you interpret your results. For more detailed protocols for blotting and cloning, see Sambrook *et al.* (1989) or other appropriate laboratory manuals.

A. Comparison of RACE Products Obtained with GSPs & NGSPs

For the 5'-RACE products, compare the products of primary amplifications performed with AP1 and GSP1 to the products obtained using AP1 and NGSP1 and the same cycling conditions and ds cDNA as a template. (For 3'-RACE, compare the products obtained from amplifications performed with AP1 and GSP2 to those obtained with AP1 and NGSP2.) This is a good test of whether multiple bands are a result of correctly primed PCR or nonspecifically primed PCR. If bands are real (i.e., the result of correct priming), they should be slightly smaller in the reaction using the nested gene-specific primer. The difference in mobility of the products should correspond to the positions of the outer and inner (nested) gene-specific primers in the cDNA structure. (**Note:** Do not use AP2 in these reactions, because it will cause a size decrease in all of the PCR products.) If you have multiple bands with AP1 and GSP1 (or GSP2), some of these may disappear upon amplification with AP1 and NGSP1 (or NGSP2).

B. Southern Blot Analysis

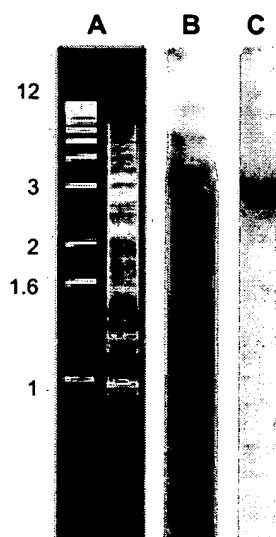
Much stronger confirmation can be obtained by probing a Southern blot of your RACE products using an internal gene-specific probe (usually one of your other GSPs). This method can be particularly useful for determining which bands are real when RACE produces multiple bands. (Multiple bands are more common with 5'-RACE than with 3'-RACE.) Figure 5 shows Southern analysis of the 5'-RACE products from the insulin-like growth factor receptor type 2 cDNA (ILGFR2). The ILGFR2 mRNA, which is large (~ 9 kb) and relatively rare, is one of the most difficult targets we have analyzed using the Marathon Kit.

XII. Characterization of RACE Products... *continued*

1. Repeat the RACE reactions and examine the products on an agarose/EtBr gel.
2. Photograph the gel, then transfer the DNA to a nylon membrane using standard blotting procedures.
3. Prepare a hybridization probe that does not have sequences in common with GSP1 (or 2). The probe can be end-labeled NGSP1 (or 2). Alternatively, if your GSPs define overlapping 5' and 3' fragments, GSP2 can be used as a probe to characterize your 5'-RACE products, and GSP1 can be used as a probe to characterize your 3'-RACE products. Nick-translated or random-primed internal restriction fragments (from a previously cloned partial cDNA) can also be used.
4. Hybridize the probe to the Southern blot, wash under moderate-to-high stringency conditions, and expose x-ray film.
5. Compare the hybridization pattern to the photograph of the agarose/EtBr gel.

Generally, you will want to isolate the RACE product(s) that correspond(s) to the largest band(s) on the Southern blot. As seen in Figure 6, there may be larger RACE products that appear on the agarose gel but do not hybridize to the gene-specific probe. These bands are generally due to nonspecific priming. Smaller bands that hybridize to your probe may be the result of incomplete reverse transcription; however, you cannot exclude the possibility that some of these shorter bands are real and correspond to alternatively spliced transcripts, transcripts derived from multiple promoters, or other members of a multigene family.

Figure 6. Identifying the correct RACE products by Southern blotting. Panel A: Agarose/EtBr gel showing the products of 5' Marathon RACE using a GSP derived from the ILGFR2 cDNA (Lane 2; expected product ~3 kb). Lane 1: 1-kb DNA ladder. Panel B: Southern blot of the gel seen in Panel A probed with a NGSP for ILGFR2. Note that the hybridization signal at the top of the blot is considerably lower than the top of the DNA smear seen in Panel A. To obtain the full-length cDNA, a second gel was run and the portion of the gel corresponding to just below the 3-kb size marker was excised. The DNA was eluted and cloned, and multiple independent clones were tested as described in the protocol to identify the largest insert derived from the ILGFR2 gene. Panel C: The same blot was reprobed with an internal gene-specific probe derived from the 5'-end of the cDNA. This confirms that the band at the top of Panel B is the correct 5'-RACE product. (Most researchers will not have the necessary probe to confirm their 5'-RACE product in this manner.)



XII. Characterization of RACE Products... *continued*

6. Once you have determined the band(s) of interest, repeat the RACE reaction, isolate the DNA from the band(s) of interest (using the NucleoTrap Gel Extraction Kit and protocol provided), and proceed with your experiments.

C. Cloning & Sequencing RACE Products

1. Purify the RACE product(s) of interest using the NucleoTrap Gel Extraction Kit and User Manual provided. In our experience, silica bead-based methods such as NucleoBond work well for RACE products up to about 2.5 kb. With longer fragments, we have obtained the best results using electroelution or DNA purification cartridges. (If you choose another method of DNA purification, resuspend your DNA in 30 µl of Tricine-EDTA Buffer.)
2. Verify recovery of the desired DNA fragment by examining 5 µl on an agarose/EtBr gel.
3. Clone the purified PCR product directly into a T/A-type PCR cloning vector. Alternatively, you may be able to clone into conventional vectors using the *Not*I, *Srf*I, *Xma*I, and *Eco*R I restriction sites in the Marathon Adaptor and/or cDNA Synthesis Primer, and restriction sites introduced in your GSP.
4. Identify clones containing gene-specific inserts by colony hybridization using a ³²P-end-labeled, nested GSP as a probe or by sequencing from your GSP. For 5'-RACE products, we recommend picking at least 8–10 different independent clones in order to obtain the maximal possible amount of sequence at the 5' end. (Reverse transcription does not always proceed all the way to the 5' end of the mRNA template [especially for long templates]. Furthermore, the action of T4 DNA polymerase removes 0–20 bases from the 5'-end.)

Once you have identified the clones containing the largest gene-specific inserts, obtain as much sequence data as you can. Ideally, you will be able to sequence the entire open reading frame, as well as 5' and 3' untranslated regions.

After RACE products have been characterized by partial or complete sequencing, you have two options for generating the full-length cDNA:

- 1) Generation of Full-Length cDNA by PCR (Section XIII).
- 2) Generation of Full-Length cDNA by Cloning (Section XIV).

XIII. Generation of Full-Length cDNA by PCR

We have used this method successfully with several transcripts in the 5–10-kb range and with several other smaller transcripts. We have had success with both abundant and relatively rare mRNAs. Please note that amplification of large cDNAs requires significantly longer extension times, as described in Step 6; however, if the extension time is too long, some smearing may be observed. Careful primer design is critical.

All Marathon PCR reactions have been optimized with CLONTECH's Advantage 2 Polymerase Mix, which includes TaqStart Antibody for automatic hot start PCR. If you choose not to use Advantage 2 Polymerase Mix, you must use your polymerase mix with some form of hot start PCR (i.e., TaqStart Antibody, wax beads, or manual hot start) to minimize background in your RACE reactions.

1. Design 5' GSP and 3' GSP primers based on the sequence obtained from your 5'- and 3'-RACE products. These primers should be derived from the 5' and 3' ends of the cDNA (as shown in Figure 4) and should be 23–28 nt long. We do not recommend adding restriction sites to the ends of your primers, as we have observed higher background in some cases. Consult the guidelines in Section V for more information on the design of the primers. In some cases it may be necessary to design nested 5' and 3' primers; however, we recommend you first try to amplify the full-length cDNA with a single pair of primers.
2. Prepare enough master mix for all PCR reactions and one extra reaction to ensure sufficient volume. For each 50- μ l PCR reaction, mix the following reagents:
 - 36 μ l H₂O
 - 5 μ l 10X cDNA PCR Reaction Buffer
 - 1 μ l dNTP Mix (10 mM)
 - 1 μ l Advantage 2 Polymerase Mix (50X)
 - 43 μ l Final volume
3. Mix well by vortexing (without introducing bubbles), then briefly spin the tube in a microcentrifuge.
4. Prepare PCR reactions as shown in Table V. Add the components in the order shown in 0.5-ml PCR tubes and mix gently.

XIII. Generation of Full-Length cDNA by PCR *continued*

TABLE V: SETTING UP PCR TO AMPLIFY FULL-LENGTH cDNA

Component	Test Tube #:	1	2 ^a	3 ^a
		Full-length	5'-RACE	3'-RACE
Adaptor-ligated ds cDNA ^b		5 µl	5 µl	5 µl
5' GSP primer (10 µM)		1 µl	1 µl	---
3' GSP primer (10 µM)		1 µl	---	1 µl
GSP1 primer (10 µM)		---	1 µl	---
GSP2 primer (10 µM)		---	---	1 µl
Master Mix		43 µl	43 µl	43 µl
Total volume		50 µl	50 µl	50 µl

^a The 5'- and 3'-RACE reactions are optional controls. The 5' and 3' GSPs are critical for the success of full-length amplification. If the full-length amplification does not work, the most likely reason is the design of these primers. These controls can help determine whether you have a problem with one of the primers.

^b Adaptor-ligated ds cDNA from Section IX. See Figure 4 for relationship of the primers.

- Overlay the contents of each tube with 2 drops of mineral oil and place caps firmly on each tube.

Note: This is not necessary if you are using a hot-lid thermal cycler.

- Commence thermal cycling using the following program:

PE DNA Thermal Cycler 480:	PE GeneAmp Systems 2400/9600 (or "hot-lid" thermal cycler):
• 94°C for 1 min	• 94°C for 30 sec
• 25 cycles:	• 25 cycles:
94°C 30 sec	94°C 5 sec
72°C 2–15 min*	72°C 2–15 min*

* The extension time in min should equal the expected length of the cDNA (to the nearest kb) plus 2 min (e.g., if your expected product is 6 kb, use 8 min [6 + 2]).

Note: you may need to determine the optimal cycling parameters for your gene empirically. If you see weak bands or no bands, perform an additional five cycles. For additional suggestions on optimizing RACE PCR conditions, refer to Section XVI.

- Analyze 5 µl of each sample on a 1.2 % agarose/EtBr gel.

Expected results: In most cases, you should see a single major product. If so, proceed to steps 8–12 for gel purification of the full-length cDNA. Gel purification is recommended (instead of cloning the PCR product directly) even though the product of this reaction is often a single strong band.

If you do not see a single major product, refer to Section XVII.

XIII. Generation of Full-Length cDNA by PCR *continued*

8. Pour a preparative 1.2% agarose gel in TAE buffer with EtBr (0.3 µg/ml).
Note: Do not use TBE buffer, as we have had difficulty cloning full-length cDNAs purified from TBE gels.
9. Load the remaining 45 µl of the PCR amplification mixture on the gel, along with appropriate DNA size markers.
10. Using a medium- to long-wave length UV light (≥ 300 nm) to visualize the DNA, cut out the band corresponding to the fused, full-length cDNA.
Note: Be careful to minimize exposure of your DNA to UV.
11. Purify the band of interest using the NucleoTrap Gel Extraction Kit and the NucleoTrap User Manual (PT3169-1). In our experience, silica bead-based methods such as NucleoTrap work well for PCR products up to about 2.5 kb. With longer fragments, we have obtained the best results using electroelution or DNA purification cartridges. (If you choose another method of DNA purification, resuspend your DNA in 30 µl of Tricine-EDTA Buffer.)
12. Clone the full-sized cDNA into a T/A-type PCR cloning vector .

In our experience, large cDNAs can be damaged during purification by exposure to UV in the presence of EtBr. If your full-length cDNA is longer than 3 kb, we suggest that you test the quality of the purified primary PCR product by repeating the reaction in Steps 4–6 using 5 µl of a 1/50 dilution of your PCR product as a template. If the DNA is damaged, reamplification will not give a single, strong band. If you cannot amplify a full-length cDNA that can be readily reamplified, we recommend that you clone the PCR product directly (i.e., without gel purification) and screen (by hybridization with a gene-specific probe) for colonies that contain full-length, gene-specific inserts.

To be certain of obtaining the correct product, we recommend that you always pick several transformants and confirm the insertion of the full-length cDNA of interest. Again, this is especially important with cDNAs larger than about 3 kb.

XIV. Generation of Full-Length cDNA by Cloning

If you have cloned overlapping 5'- and 3'-RACE products, and if there is a restriction site in the overlapping portion of the cDNA sequence, it is fairly easy to generate the full-length cDNA by standard cloning techniques. (Note that restriction sites introduced with your GSP are not suitable for this purpose, since using such a site to fuse your 5' and 3' fragments would, in most cases, introduce foreign sequence into the middle of your cDNA. For the same reason, do not fuse your 5' and 3' cDNA fragments using restriction sites in polylinkers adjacent to your cloned RACE products.) Simply digest each fragment with the enzyme, and join them using T4 DNA ligase. Clone the resulting full-length cDNA into the vector of your choice using the restriction sites introduced by the Marathon Adaptor (which is on both ends of full-length cDNAs created in this manner) and the Marathon cDNA synthesis primer (on the 5' end). The Marathon Adaptor contains sites for *Not* I and *Xma* I (sticky ends) and *Srf* I (blunt ends), while the cDNA Synthesis Primer contains *Not* I and *Eco*R I sites (See Appendix A). This facilitates easy directional cloning of the *Srf* I/*Not* I, *Srf* I/*Eco*R I, *Xma* I/*Not* I or *Xma* I/*Eco*R I fragments, or non-directional cloning of *Not* I/*Not* I or *Srf* I/*Srf* I fragments into suitable vectors. *Srf* I and *Not* I are extremely rare in mammalian genomes, occurring approximately once in 10⁶ bp, and hence are unlikely to be present in most cDNAs. Alternatively, if you are working directly with the products of your 5'- and 3'-RACE reactions, you can clone the full-sized cDNA directly into a T/A-type PCR cloning vector.

XV. Troubleshooting of ds cDNA Synthesis

A. Low yield of experimental ds cDNA

If the gel run in Step VIII.25 indicates that the yield of your experimental ds cDNA is low in comparison with the ds cDNA produced from the control poly A⁺ RNA, but the size distribution is good, you may want to use a more concentrated dilution of adaptor-ligated ds cDNA. If you do not generate enough cDNA to get an efficient amplification of a 5'-fragment and/or 3'-fragment of the cDNA of interest, try to concentrate experimental RNA by ethanol precipitation and repeat ds cDNA synthesis.

B. If ds cDNA appears as a smear of 1–2 kb

If your experimental ds cDNA appears as a smear that is no larger than 1–2 kb, the RNA may be impure or degraded. Examine the poly A⁺ used as a starting material on a denaturing formaldehyde 1% agarose/EtBr gel. Intact mammalian poly A⁺ RNA should appear as a smear (usually 0.5–9 kb) with faint 28S RNA and 18S RNA bands at 4.5 and 1.9 kb. The size distribution may be considerably smaller (0.5–3 kb) for nonmammalian species (e.g., plants, insects, yeast, amphibians, etc.). Compare the smear from your experimental RNA sample to 2–3 µl of the Control Poly A⁺ RNA provided. If your experimental poly A⁺ RNA appears significantly smaller than expected (e.g., no larger than 1–2 kb), we suggest you prepare fresh RNA after checking your RNA purification reagents for RNase and other impurities. If problems persist, you may need to find another source of tissue/cells. Alternatively, you may wish to use one of CLONTECH's Poly A⁺ RNAs. The yield and efficiency of cDNA synthesis depend on the ratio between AMV Reverse Transcriptase and RNA.

The optimal concentration of poly A⁺ for first-strand cDNA synthesis is 100–200 µg/ml. Using a lower concentration of RNA can reduce size distribution of synthesized cDNA products.

C. If you cannot obtain satisfactory results in RACE reactions

If you cannot obtain satisfactory results in your subsequent RACE experiments, after performing the PCR Control Experiment (Section X), and following the troubleshooting suggestions for 5'- & 3'-RACE (Section XVI), you may wish to repeat the cDNA synthesis using either a GSP or random hexamers instead of the cDNA Synthesis Primer provided with the kit. This is most likely to be useful when working with large and/or rare cDNAs with "difficult" 5' ends.

XVI. Troubleshooting RACE Reactions

It is generally advisable—and often necessary—to optimize your 5'- and 3'-RACE reactions. This generally consists of improving the yield of your desired fragment(s), while decreasing the amount of background or nonspecific and/or incomplete bands in your RACE reactions. The cDNA synthesis protocols contained in this User Manual typically produce enough adaptor-ligated ds cDNA for 100 or more RACE PCR reactions, and the Marathon Kit contains enough primers and dNTP Mix for 100 PCR amplifications. Thus, there is plenty of material for optimizing your RACE reactions at this stage.

Tables III and IV in the User Manual include several controls that will help you troubleshoot the reaction if yields are suboptimal. These include:

- **Tube # 2:** 5'- or 3'-RACE PCR using the positive control TFR Primer, the AP1 Primer, and the ds cDNA made from the Control Poly A⁺ RNA. Figure 1 in the Introduction shows the results of 5' and 3' RACE using these controls, and an additional set of primers for the human actin.

A smear of large molecular weight material may appear at the top of some lanes. As discussed in Appendix A, the upper limit of the suppression PCR effect is about 6 kb. The large, nonspecific amplification products that do appear in some Marathon experiments generally do not interfere with interpretation of RACE results; however, care must be taken to avoid these products when cloning and otherwise using your RACE products for subsequent experiments.

- **Tube # 3:** An additional positive control using both GSPs to amplify your adaptor-ligated ds cDNA. If you do not have suitable 5'- and 3'-GSPs (i.e. GSPs that create overlapping 5'- and 3'-RACE products), use the control 5'- and 3'-RACE TFR Primers with 5 µl of your positive control adaptor-ligated cDNA or 5 µl of the PCR Control cDNA provided with the kit. This should give a single band corresponding to the overlap between the primers. This result confirms that your cDNA, or the TFR cDNA, is present in (and can be amplified from) your adaptor-ligated ds cDNA.
- **Tube # 4:** A negative control using AP1 by itself to amplify your adaptor-ligated ds cDNA. With less than 30 cycles, this should produce no product. (AP1 may produce some large [5–8 kb] smear product with higher cycle numbers; RACE products can generally still be seen in the presence of these bands.) If this control produces a smear or ladder of extra bands, you may need to alter the cycling parameters or perform a secondary amplification using the AP2 Primer.
- **Tube # 5:** A negative control using each GSP by itself. This control should produce no product. If this control produces a smear or ladder of extra bands, you may need to alter the cycling parameters, perform a secondary amplification using nested primers, or redesign your original primer.

XVI. Troubleshooting RACE Reactions *continued*

A. General Considerations.

- **Troubleshooting GC-rich templates:** If your PCR product is not the expected size, especially your 5'-RACE product, this may be due to difficulty amplifying a GC-rich template. CLONTECH offers the Advantage-GC cDNA Polymerase Mix and PCR Kit for efficient amplification of GC-rich templates. However, when using this polymerase mix or kit, the master mix recipes will need to be modified to include GC-Melt™ and for the 5X PCR Reaction Buffer, instead of a 10X buffer supplied with most polymerases. Additionally, the PCR parameters may need to be optimized for these templates. We recommend that you perform the initial RACE reactions with Advantage 2 Polymerase Mix, then perform the RACE reactions using the Advantage-GC Polymerase Mix to confirm the product is the same size in both reactions.
- **High-fidelity PCR:** If you are going to sequence or clone your RACE products for further analysis, we recommend performing your RACE reactions using the Advantage-HF PCR Kit. The Advantage-HF PCR Kit is designed to yield products of less than 2.5 kb with fidelity comparable to the leading high fidelity polymerase. This kit may not be ideal for cDNA templates that are greater than 2.5 kb, but it is especially well suited for applications in which the RACE product will be sequenced or cloned for use in additional experiments. Again, the initial RACE reactions should be performed using the Advantage 2 Polymerase Mix to confirm the product is present and that the GSPs work well.
- **Troubleshooting touchdown PCR:** When troubleshooting touchdown PCR, we recommend that you begin by modifying the final (third) set of cycle parameters (i.e., the 20–25 cycles performed at 68°C in Program 1). If you do not observe an amplified product after 20 cycles at 68°C, run five additional cycles. If the product still does not appear, add an additional 3–5 cycles at 68°C or try performing a new PCR experiment with 25 cycles of 65°C for 30 sec, 68°C 4 min. The last program is especially useful if you suspect that your GSP has a T_m close to or less than 70°C.
- **Adapting the Marathon protocol for thermal cyclers other than the Perkin-Elmer DNA Thermal Cycler 480 or GeneAmp Systems 2400/9600:** As noted elsewhere in this manual, cycling parameters in this protocol must be optimized if you are using a thermal cycler other than the Perkin-Elmer DNA Thermal Cycler 480 or GeneAmp Systems 2400/9600. If you have access to one of these thermal cyclers, we strongly recommend that you use it.

B. No bands are observed in your positive control (GSP1 + GSP2).

The control PCR reaction using your sense and antisense GSPs and your adaptor-ligated ds cDNA to amplify the internal fragment of your gene is very important. If this reaction fails to produce the expected internal cDNA fragment, there are at least four possible explanations:

- There may be a problem with your 50X polymerase mix. If you have not already done so, perform the control PCR reaction described in Section X.

XVI. Troubleshooting RACE Reactions *continued*

If you are not using Advantage 2 Polymerase Mix, consider switching. The Marathon protocol was optimized with Advantage 2 Polymerase Mix.

- Your gene may be expressed weakly or not at all in your starting RNA. It may be necessary to find a new source of RNA. The efficiency of both 5'- and 3'-RACE amplification depends on the abundance of the experimental poly A⁺ RNA.
- There is a problem with your primers. This could be due either to poor primer design or poor primer preparation. First try lowering your annealing/extension temperature. If this does not work, you may need to design new primers or repurify your GSPs to remove impurities.
- Your RNA was degraded or you failed to get full-length cDNA synthesis (usually because AMV-RT cannot copy the full-length cDNA due to strong secondary structure and/or high GC-content). This is indicated if the 3'-RACE works, but the 5'-RACE does not, and the positive control (GSP1 + GSP2) does not produce the expected fragment.

You may be able to obtain more information by amplifying the internal fragment (using GSP1 and GSP2) using genomic DNA as the template. If this produces the expected band, this indicates that your primers are usable and the problem is either (a) the target RNA is a poor template for AMV RT; or (b) the RNA is not expressed in the tissue source you have chosen. Note, however, that this is not a conclusive test, since your primers may be separated by an intron in the genomic DNA. If this is the case, amplification of genomic DNA will give a larger fragment than expected or no fragment at all.

C. No bands are observed with the experimental or control cDNA samples.

- Confirm that your 50X polymerase mix is active by repeating the positive control RACE PCR experiment (Section X).
- The complete absence of RACE products (especially in *both* 5'- and 3'-RACE reactions) may indicate failure of adaptor ligation (assuming you have obtained the expected result in the positive control PCR experiment [Section X]). In this case, try repeating the adaptor ligation and RACE protocol.

Failure of adaptor ligation is especially likely if: (a) the size and yield of experimental and positive control ds cDNA looked good at Step VIII.25; and (b) the positive control using sense and antisense GSPs produced the expected internal cDNA fragment.

D. No bands are observed with the experimental cDNA sample, but the TFR positive control gives the expected product.

- If the yield or size distribution of ds cDNA products was low in comparison with the control cDNA at Step VIII.25, read the troubleshooting recommendations for ds cDNA product (Section XV).

XVI. Troubleshooting RACE Reactions *continued*

- If the yield and size distribution of the ds cDNA were normal compared to the positive control at Step VIII.25, there are several things you can try:
 1. Increase the cycle number.
 2. Lower the annealing temperature by increments of 2–5°C.
 3. Increase the amount of adaptor-ligated ds cDNA used. If you have some of the undiluted adaptor-ligated ds cDNA, you can prepare a second, more concentrated dilution.
 4. Generate different GSPs.
 5. Try a new source of RNA.
 6. If 5'-RACE gives no product, but 3'-RACE works well, you may want to try repeating ds cDNA synthesis using a GSP or random hexamers instead of the cDNA Synthesis Primer provided with this kit.

E. RACE cDNA product consists of multiple bands.

In many cases, your initial experiments will produce multiple 5'- and/or 3'-RACE products. As mentioned above, you will have to determine which are real and which are artifacts. While the following guidelines will help you eliminate artifacts, confirmation of real and complete bands will require additional studies such as mapping of transcription start sites, intron/exon structure and polyadenylation sites, and genomic sequencing.

Multiple fragments do not mean you cannot proceed with generating the full-length cDNA, however, you may save time in the long run if you try to eliminate nonspecific fragments by troubleshooting the reactions. If multiple fragments persist and you want to proceed, you generally will want to start with the largest fragment from each RACE reaction, since it is most likely to be a true, complete RACE product.

Sources of “real” multiple RACE products

Individual genes can give rise to multiple sizes of transcripts—and hence to multiple RACE fragments—via at least three mechanisms:

- Alternative splicing can cause multiple products in either 5'- or 3'-RACE.
- Use of different transcription initiation sites causes multiple 5'-RACE products.
- Use of different polyadenylation sites causes multiple 3'-RACE products.

Alternatively, the gene may be a member of a multigene family, in which case your “gene-specific” primers may simultaneously amplify several highly homologous cDNAs.

Distinguishing true polymorphic forms of an RNA is a matter for scientific investigation. It may be possible, however, to find an alternative source of RNA in which one form is more abundant than others.

XVI. Troubleshooting RACE Reactions *continued*

Sources of artifacts

Multiple bands often do not correspond to actual and complete transcripts. These artifact RACE products can be divided into two classes—incomplete and nonspecific.

There are several possible sources of incomplete fragments, which are generated from correctly primed sites.

- Premature termination of first-strand cDNA synthesis caused by pausing of RT generally causes multiple 5'-RACE products. This is a common problem with larger RNAs, and it is a difficult problem to overcome, since it is due to an intrinsic limitation of RT.
- Degradation of the RNA used as starting material generally causes multiple 5'-RACE products.
- Difficulty in amplifying certain “difficult” genes can cause multiple products in either 5'- or 3'-RACE; often a result of high GC content.

Nonspecific RACE products arise from nonspecific binding of the primer to multiple sites in the ds cDNA or primer-dimer artifacts.

Suggestions:

- If you have not already done so, repeat your RACE reactions with all of the recommended controls. In particular, be sure that your GSPs do not give bands when used alone, and that they give a single band when used together. If either GSP alone gives persistent bands, we recommend altering the cycling parameters or designing nested primers as discussed below. Also repeat the Positive Control RACE PCR Experiment.
- If you have not already done so, repeat your RACE reactions using some form of hot start PCR (antibody-mediated, wax beads, or manual).
- Repeat your reactions using 5 µl of a 5–10-fold lower dilution of the adaptor-ligated ds cDNA.
- If you have not already done so, examine the size distribution of your RNA starting material and your ds cDNA as discussed in Section VIII. If either RNA or ds cDNA looks smaller than expected, repurify your RNA and repeat cDNA synthesis and adaptor ligation.
- If multiple bands persist, try altering the PCR cycling parameters:
 - a. Increase the stringency of your PCR by raising the annealing temperature in increments of 2–5°C. In many cases, bands arising from nonspecific priming will disappear while real or incomplete products will persist.
 - b. Reduce the cycle number. Again, bands arising from nonspecific priming may disappear, while real or incomplete products will persist.

XVI. Troubleshooting RACE Reactions *continued*

- c. Reduce the extension time.
- d. In the case of large RACE products, increasing the extension time may help eliminate extra bands.
- If multiple bands persist, try designing a new set of primers:
 - a. Redesign your primers so that they have a T_m greater than 70°C and use the cycling parameters for touchdown PCR.
 - b. We recommend that you design new primers that will give RACE products that are slightly different in size than those expected with the original primers. These new primers can either be used by themselves or in combination with the original primers in “nested PCR”. In nested PCR the product of a PCR reaction is reamplified using a second set of primers that is internal to the original primers. This often greatly reduces the background and nonspecific amplification seen with either set of primers alone. The design of nested primers is discussed in Section V.
 - c. Prior to performing nested RACE PCR, we recommend that you perform separate primary amplifications with AP1 and either the GSP1 or NGSP1. This is a good test of whether multiple bands are a result of correctly primed PCR or nonspecifically primed PCR. If the multiple bands are real (i.e., the result of correct priming), they should be present in both reactions, but slightly smaller in the reaction using the nested primers. The difference in mobility of the products should correspond to the positions of the GSP and NGSP in the cDNA structure.

If none of the above suggestions work, you may want to try repeating ds cDNA synthesis using a GSP or random hexamers instead of the cDNA Synthesis Primer provided with this kit.

F. RACE cDNA product is smeared.

Note: Some Marathon reactions produce very complex patterns of bands that appear almost as smears. See Figure 5 for an example. Section XIII gives some guidelines for interpreting these complex patterns and isolating the band(s) of interest.

In most cases of true smearing, a problem has occurred prior to the RACE reaction. *This is especially true if the 3'-RACE reaction produces a smear*; smearing of the 5'-RACE reaction products *only* may indicate a difficult template for reverse transcription or degraded RNA. Smearing of *both* reactions is a strong indication of contamination of your starting RNA, a problem in reverse transcription or second-strand cDNA synthesis, or a problem in adaptor ligation. In these cases we recommend repeating the entire procedure after repurifying your RNA (or confirming that your RNA is intact and clean).

XVII. Troubleshooting Generation of Full-Length cDNA by PCR

If it appears that smearing is not due to a problem that occurred prior to RACE, you can try optimizing your RACE reactions using the troubleshooting tips described above for multiple bands.

In most cases, you should see a single major product of the size predicted from Northern analysis or analysis of your 5'- and 3'-RACE products. If you do not observe a single, major band and if you cannot resolve your full-length cDNA by optimizing the extension time and cycle number, we suggest that you design additional (nested) 5' and 3' GSPs. Most problems with the full-length PCR reaction are due to poor primers and can be corrected simply by using better primers. If one of the controls in Table V also does not work, try that nested primer first. Try additional primary PCR reactions with different combinations of flanking primers (i.e., 5' GSP and 3' NGSP; 5' NGSP and 3' GSP; 5' NGSP and 3' NGSP). If that doesn't work, then try nested PCR.

See Section XVI for additional suggestions on interpreting your results and optimizing your PCR reactions

XVIII. References

Marathon cDNA amplification has been cited in over 140 research articles. For a complete list of citations, see CLONTECH's web site (<http://www.clontech.com>).

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XIX. Related Products**Cat. #**

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Appendix A: Marathon cDNA Adaptor & Primers

The Marathon cDNA Adaptor has three design features that are critical to the success of Marathon cDNA amplification:

- The use of a 5'-extended adaptor that has no binding site for the AP1 Primer used in primary PCR. An AP1 binding site can only be generated by extension of the gene-specific primer (GSP).
- Blocking of the exposed 3' end of the adaptor with an amine group to prevent extension of the 3' end (which would create an AP1 binding site and allow nonspecific amplification).
- The use of an adaptor primer that is shorter than the adaptor itself (suppression PCR). As shown in Figure 7, the suppression PCR effect prevents amplification of templates where the 3' end has been extended to create an AP1 binding site. Though rare, such extension does occur, presumably due to incomplete amine modification or incomplete adaptor ligation. Given the exponential nature of PCR amplification, such events would lead to nonspecific amplification and unacceptable backgrounds in the absence of suppression PCR.

Each of these features helps eliminate nonspecific amplification among the general population of cDNA fragments. Together, they allow amplification of a specific target from a very complex mixture of DNA fragments—all of which have the same terminal structure—using a single set of GSPs.

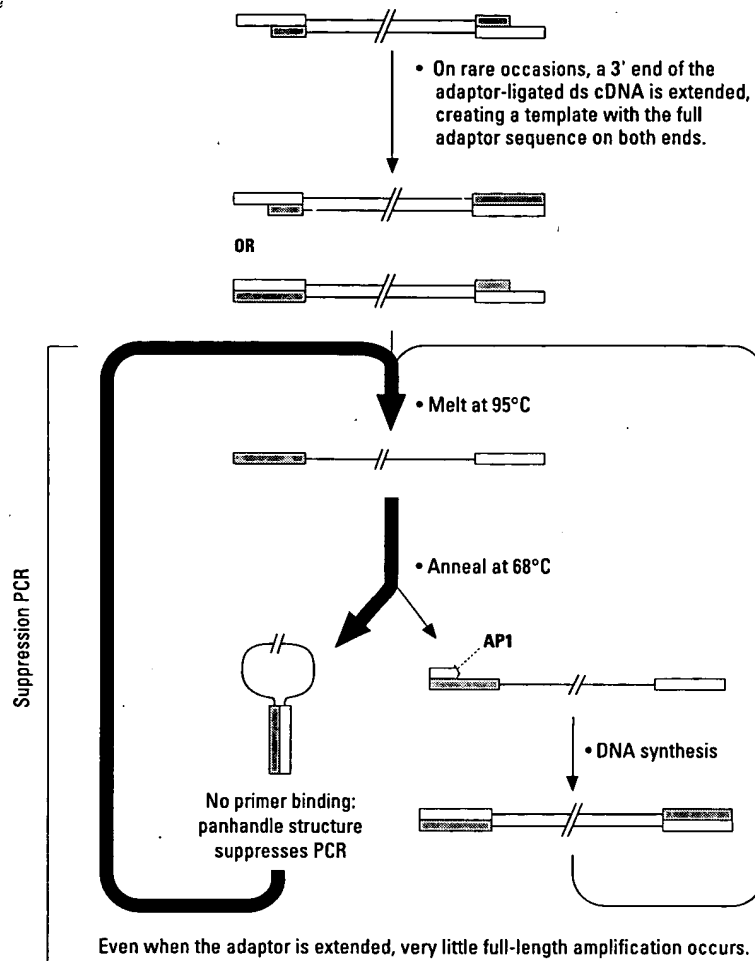
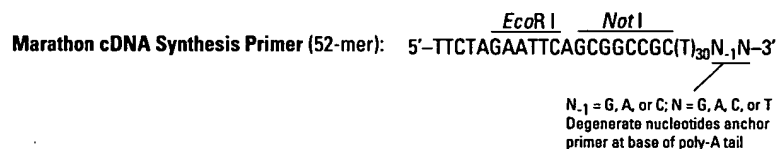
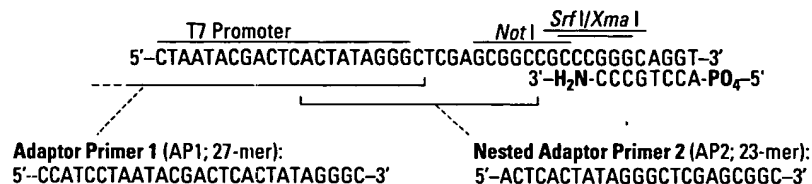
Appendix A: Marathon cDNA Adaptor & Primers... *continued*

Figure 7. The suppression PCR effect. In rare cases, the 3' end of the Marathon Adaptor gets extended. (Though rare, such extension does occur, presumably due to incomplete amine modification during oligonucleotide synthesis or incomplete adaptor ligation.) This creates a molecule that has the full-length adaptor sequence on both ends and can serve as a template for end-to-end amplification. Without suppression PCR, these rare events would lead to unacceptable backgrounds in RACE reactions due to the exponential nature of PCR amplification. However, in suppression PCR, the adaptor primer is much shorter than the adaptor itself. Thus, during subsequent thermal cycling, nearly all the DNA strands will form the "panhandle" structure shown above, which cannot be extended. At the appropriate annealing/extension temperature, this intramolecular annealing event is strongly favored over (and more stable than) the intermolecular annealing of the much shorter adaptor primer to the adaptor.

Appendix A: Marathon cDNA Adaptor & Primers... *continued*

Marathon cDNA Adaptor:

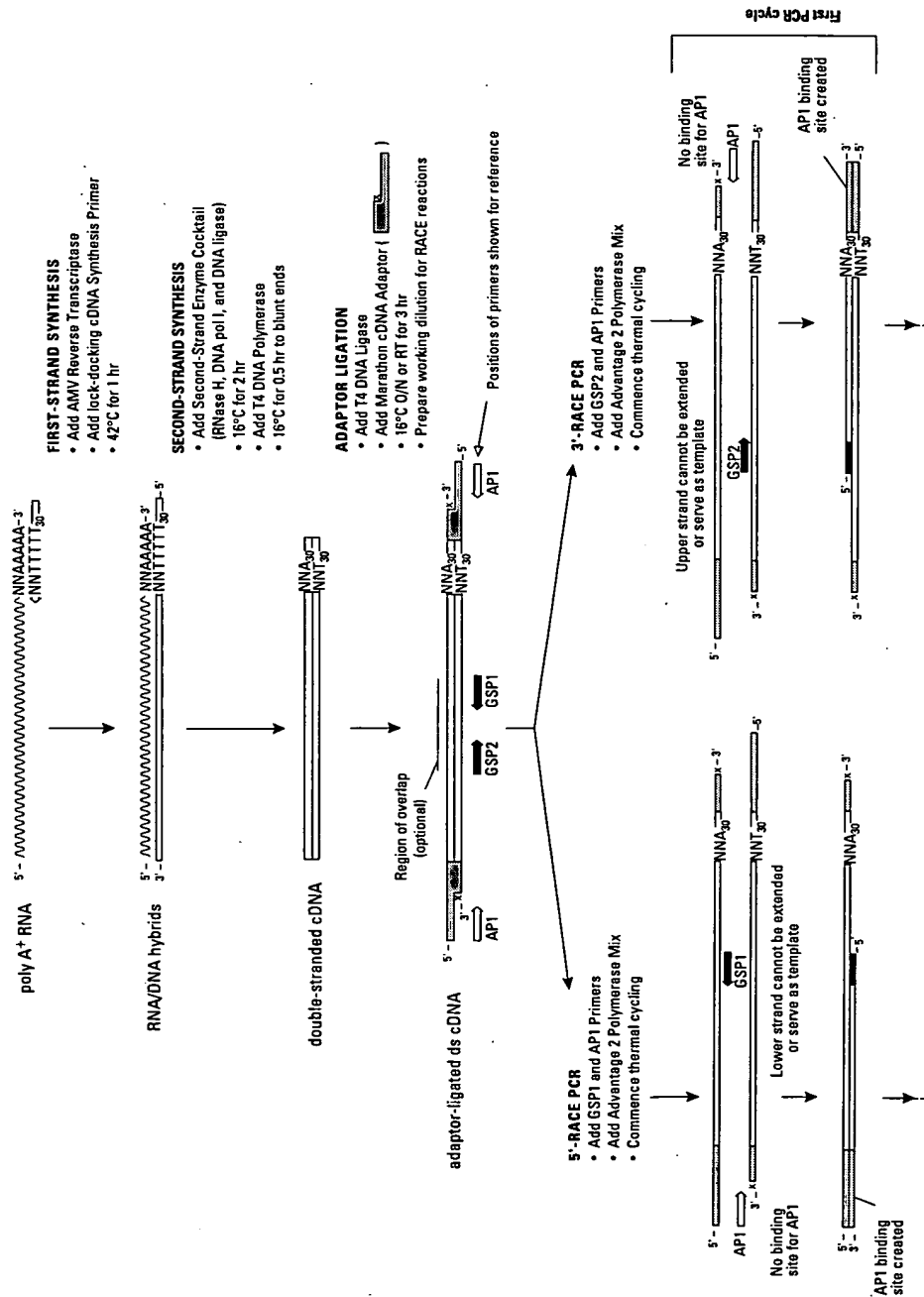


5'-RACE TFR Primer (24-mer): 5'-GTCAATGTCCCAAACGTACCAGA-3'

3'-RACE TFR Primer (29-mer): 5'-ATTCGGGAATGCTGAGAAAACAGACAGA-3'

Figure 8: Sequences of the Marathon cDNA Adaptor & Primers. The T_m 's of AP1 and AP2 are 71°C and 77°C, as determined by nearest neighbor analysis (Freier *et al.*, 1986). Note, however, that only 22 of the 27 nt in AP1 bind the Adaptor during the first cycle of PCR, so the effective T_m of AP1 may be actually several degrees lower. The lower effective T_m of AP1 is the reason touchdown PCR works well with Marathon RACE reactions.

Appendix B: Detailed Flow Chart of Marathon Procedure



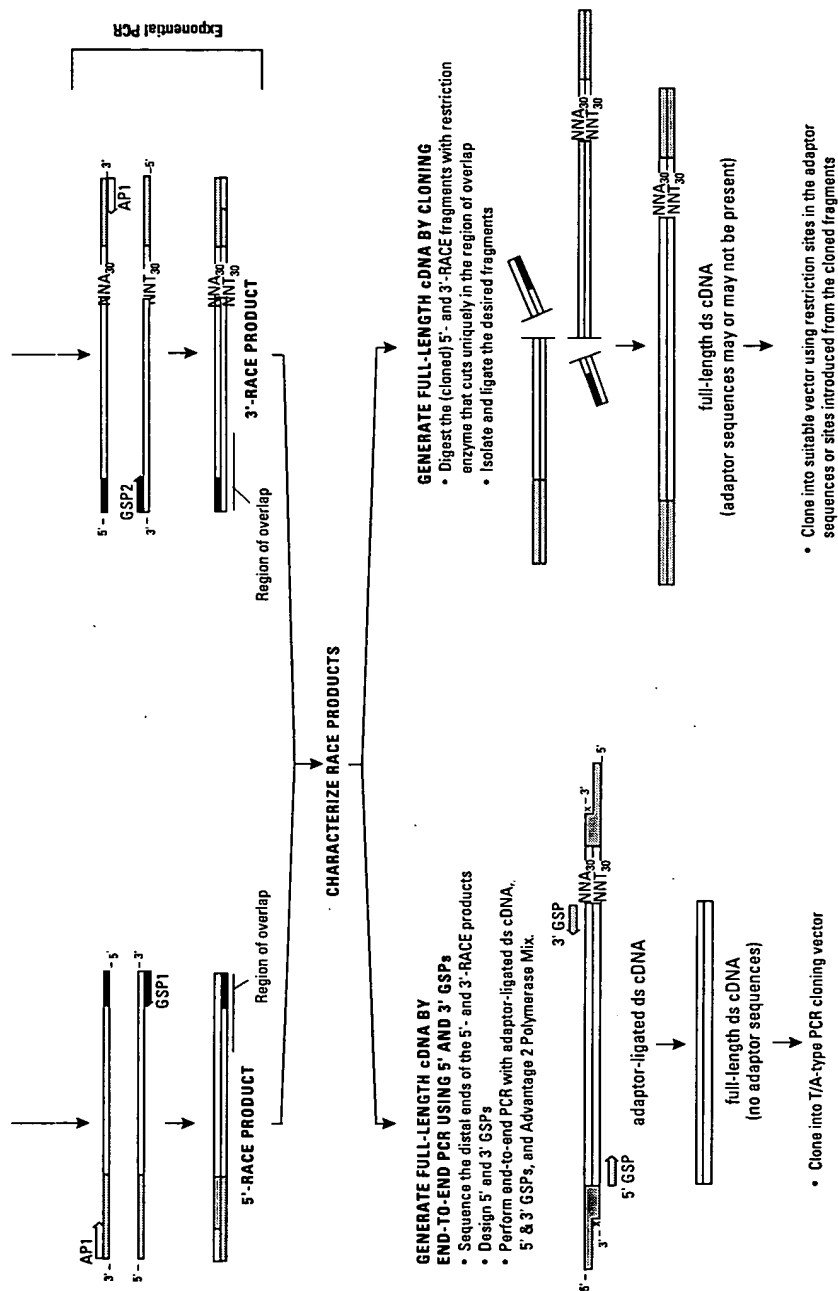
Appendix B: Detailed Flow Chart of Marathon ... *continued*

Figure 8. Detailed flow chart of Marathon cDNA amplification protocol.